DIVERSITY AND DISTRIBUTION OF BACTERIAL COMMUNITIES IN DIOXIN-CONTAMINATED SEDIMENTS FROM THE HOUSTON SHIP CHANNEL

A Thesis

by

ANNE-SOPHIE CHARLOTTE HIEKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Oceanography

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Approved by:

Chair of Committee,	Robin Brinkmeyer
Committee Members,	Peter Santschi
	John Schwarz
	Suresh Pillai
Head of Department,	Piers Chapman

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ABSTRACT

Diversity and Distribution of Bacterial Communities in Dioxin-Contaminated Sediments from the Houston Ship Channel. (May 2008)

Anne-Sophie Charlotte Hieke, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Robin Brinkmeyer

The Port of Houston and the Houston Ship Channel (HSC) are highly industrialized areas along Galveston Bay, Texas. The HSC is highly polluted with a host of persistent organic pollutants, including dioxins. The main objective of this study was to determine the potential for *in situ* bioremediation in the HSC sediments. Our study focused on the bacterial group *Dehalococcoides*, since it is the only known group to reductively dechlorinate dioxins. Culture independent methods were used to determine the presence or absence of *Dehalococcoides* in HSC sediments. Molecular methods including PCR, cloning, restriction enzyme digest, and sequencing were used to determine the diversity of *Dehalococcoides* as well as total bacterial diversity in HSC sediments. The metabolically active members of the microbial community in HSC sediments were also determined using the same molecular methods as described above.

Dehalococcoides was detected in every sediment core and at various depths within each core. Depths ranged from 1cm (SG-6) to 30cm (11261). *Dehalococcoides* diversity was centered on *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1. Overall bacterial diversity in HSC sediments was dominated by

Proteobacteria, especially *Deltaproteobacteria*, and *Chloroflexi*, which include *Dehalococcoides*. Total bacterial diversity at a wetlands control site was dominated by *Betaproteobacteria* and *Acidobacteria*. *Deltaproteobacteria* and *Chloroflexi* were determined to be the major metabolically active groups within the HSC sediments. These findings indicate that the HSC sediments have great potential for successful *in situ* bioremediation. These results also support the use of *Dehalococcoides* as a biological proxy for dioxin contamination.

DEDICATION

I would like to dedicate my thesis to my parents, my sister, and my fiancé for their unwavering love and support.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Brinkmeyer, and my committee members, Dr. Santschi, Dr. Schwarz, and Dr. Pillai, for their guidance and support throughout the course of this research.

Thanks also go to my friends and fellow lab mates and the department faculty and staff for making my time at Texas A&M University a great experience. I also want to extend my gratitude to the Texas Sea Grant College Program, which provided the majority of the funding for this research, and the Texas General Land Office/Coastal Management Program.

Finally, thanks to my mother and father for their encouragement and to my fiancé for his patience and love.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

CHAPTER I

INTRODUCTION

I. 1. Introduction

I. 1. 1. Houston Ship Channel

The Houston Ship Channel (HSC), located in the San Jacinto River Basin, in the northwest corner of Galveston Bay, Texas, is 50 miles in length, extending from the Port of Houston to the Gulf of Mexico (Fig. 1). The Port of Houston is the sixth largest seaport in the world and handles more foreign water-borne tonnage than any other U.S. port. The Port of Houston generates over $10 billion annually and each year more than 6,300 vessels pass through the HSC. The HSC is also home to the largest petrochemical complex in the United States and the second largest worldwide (43).

FIG 1. Texas, Galveston Bay, and the Houston Ship Channel (64).

This thesis follows the style of Applied and Environmental Microbiology.

The HSC is continually being dredged and the dredged sediment is used to create spoil islands and wetlands. Both the HSC and upper Galveston Bay (GB) are highly polluted with dioxins, dioxin-like compounds, and many other contaminants, such as hydrocarbons, from industrial and municipal effluents and runoff, as well as from atmospheric wet and dry deposition. In 1990, dioxins were detected in fish and crab tissue obtained from the HSC. A seafood consumption advisory for catfish and blue crabs was issued for the HSC and upper GB, and remains in effect to this day. Subsequently, the HSC was placed on the §303 (d) list of impaired water bodies as required by the 1977 Clean Water Act (as amended, 1996) and a total maximum daily loads (TMDL) study was initiated by the Texas Commission on Environmental Quality (TCEQ). The study revealed that Toxic Equivalent (TEQ) concentrations in water ranged from 0.10 to 3.16 pg TEQ/L and in bottom sediments from 0.9 to 139.8 ng/kg dry wt. (57). On average, dioxin concentrations exceeded the Texas Surface Water Quality Standard (0.093 pg/L) in more than 80% of all samples (48). The study also revealed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the major contributor to total TEQs in all samples. The entire HSC is contaminated with dioxins and recent dioxin inputs to the HSC continue despite regulatory efforts (64).

I. 1. 2. Dioxins

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (dioxins) are persistent environmental contaminants. Dioxins cause a variety of biochemical, immunological, and reproductive effects in animals and are suspected

carcinogens (6, 7, 28, 42, 46, 51). Dioxins bioaccumulate in the aquatic and terrestrial food chains posing significant and persistent risks to human health. The estimated half-life of dioxin in the human body is 7-8 years (58). Primary sources of dioxins include the production of herbicides (56), paper and pulp bleaching, metal smelting, and waste incineration (16, 18, 54, 62). Dioxins and dioxin-like compounds are hydrophobic and therefore have a high particle and lipid affinity. Their water solubility is estimated to be 19.3 ng/L (58). Due to their high hydrophobicity, dioxins present in the water column rapidly partition to organic carbon fractions (i.e black carbon) in suspended soils and can subsequently be buried in sediments (11, 38, 52). Re-suspension of polluted sediments may re-introduce dioxins into the aquatic food chain; however, this process has not been thoroughly investigated. From both fiscal and environmental perspectives, in situ microbial remediation of dioxins in the HSC and GB is preferable to alternatives, such as removal of contaminated sediments to landfills or chemical treatments. Microbial remediation would also not interfere with the vessel traffic through the HSC. Since the HSC is tidally influenced, dioxin contamination has been transferred up- and downstream of the channel, increasing the urgency of remediation.

I. 1. 3. Microbial Dechlorination

Studies of microbial dechlorination of polychlorinated compounds have been mostly limited to freshwater systems and have indicated that degradation rates are enhanced under anaerobic, reducing conditions (1-Adriaens and Grbic-Galic, 1994). Quensen et al. (44) showed that the chlorinated compound DDE (1,1-dichloro-2,2,-bis(p-

chlorophenyl)ethylene), a commercial by-product in DDT formulations, is preferentially degraded under methanogenic and sulfidogenic conditions. Another study found that 2,3,7,8-TCDD was degraded up to 86% under anaerobic, reducing conditions (25) (Fig. 2). The reductive dehalogenation of chlorinated aromatic compounds has been identified as an energy-yielding process in a number of anaerobic bacteria (22). These anaerobic bacteria use polychlorinated compounds as electron acceptors and hydrogen as an electron donor (2, 14, 22).

The reductively dechlorinating bacteria known to date belong to the low GC Gram-positive bacteria (*Desulfitobacterium* and *Dehalobacter*) or to the Proteobacteria (for example, *Desulfomonile*, *Desulfuromonas* and *Dehalospirillum*) (22, 33). Another bacterial group, *Dehalococcoides*, is also known to reductively dechlorinate highly chlorinated compounds, making the resulting congeners and other biproducts more susceptible to degradation by other bacterial groups.

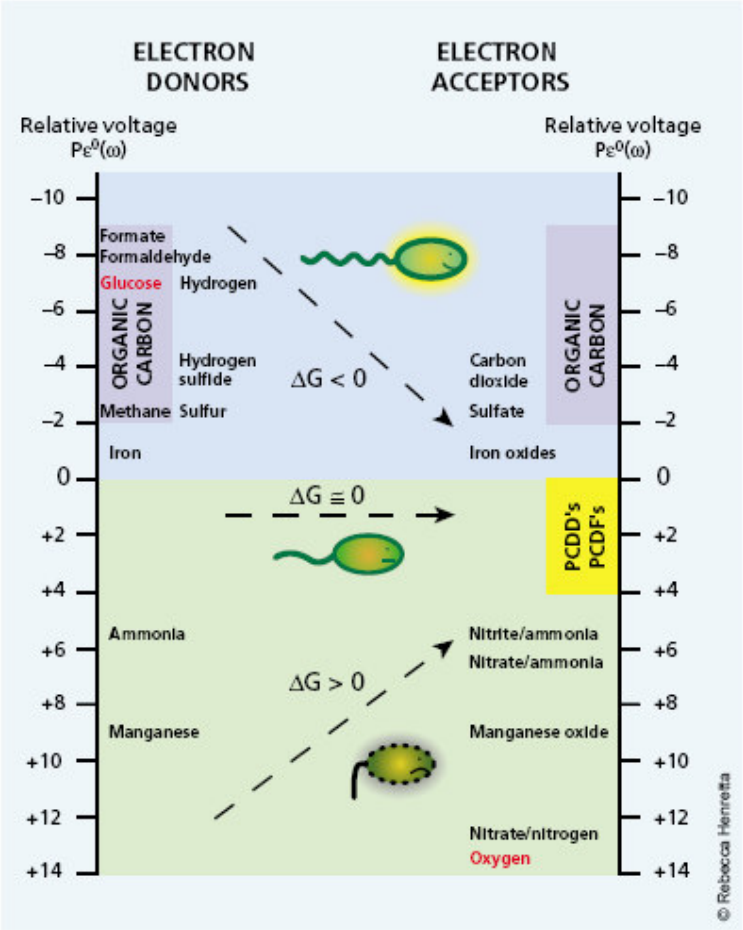

FIG 2. 'The energetic logic of life'. The relative positions, expressed as electron potential (pE), of electron donors ('edibles') and electron acceptors ('breathables') (40).

The closest phylogenetic affiliation of *Dehalococcoides* is with the green non-sulfur bacteria (20, 24, 60); however there is increasing evidence that they may constitute a new division of bacteria (22, 33). Thus far, *Dehalococcoides* have only been isolated from groundwater and other freshwater systems. *Dehalococcoides ethenogenes* strain 195, isolated from contaminated groundwater, is the only known isolated organism capable of fully dechlorinating tetrachloroethene (PCE) and other chloroethenes to the non-toxic end-product ethene (2). Strains FL2 (isolated from a highly enriched PCE-to-

ethene dechlorinating mixed culture from Red Cedar River sediment, Michigan, Loeffler et al, 2000) and DCEH2 (isolated from a dechlorinating enrichment mixed culture, GenBank accession number AJ249262) also dechlorinate chloroethenes (20). Strain CBDB1 (isolated from an enriched chlorobenzene-dechlorinating mixed culture from Saale River sediment, Germany) dechlorinates trichlorobenzenes and tetrachlorobenzenes to dichlorobenzenes, but is unable to dechlorinate PCE or trichloroethene (2). Strain CBDB1 is also able to dechlorinate chlorinated benzenes (14). Members of *Dehalococcoides* have also been shown to dechlorinate commercial polychlorinated biphenyls (PCBs) (i.e Aroclor 1260) (9). Bedard (9) also found that *Dehalococcoides* obtain energy for growth from dechlorination. In 2003, Bunge et al (14) showed that strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD). Since the dehalogenation of dioxin by *Dehalococcoides* is orders of magnitude faster (weeks versus 1 to 4 years) than its anaerobic co-metabolic reduction (1, 14) its presence in or addition to dioxin contaminated areas is a significant contribution for bioremediation (1, 2, 14, 22).

I. 1. 4. Research Objectives

My thesis research is part of a larger effort to study the fate and impacts of persistent pollutants, such as dioxins in the HSC. We are also trying to elucidate the parameters and processes required to microbially dechlorinate dioxins in HSC sediments. For my

Masters thesis, I proposed to determine the potential for bioremediation in the Houston Ship Channel. This goal was accomplished through four objectives.

Objective 1

I screened contaminated sediments from the HSC for the presence or absence of the bacterial group *Dehalococcoides,* since it is known to reductively dehalogenate dioxins and other chlorinated compounds such as chloroethenes and chlorobenzenes. *Dehalococcoides* are substrate specific bacteria and use dioxins and similar compounds exclusively as their energy source. This characteristic makes them a good indicator for dioxin contamination in the sediment.

> *Hypothesis 1:* I expect to find the bacterial group *Dehalococcoides* at higher dioxin concentrations in the sediments, but not at lower concentrations.

I tested this hypothesis by comparing the presence or absence of *Dehalococcoides* to dioxin concentrations. I extracted community DNA from sediment samples and performed PCR, first with general bacterial primers and then with *Dehalococcoides*-specific primers. PCR products were visualized on agarose gels stained with ethidium bromide.

Dioxin concentrations were also measured for certain sediment samples and these results were compared to the PCR results.

Objective 2

I constructed 16S rRNA gene clone libraries to determine total bacterial diversity as well as the diversity of dioxin-degrading bacteria, especially *Dehalococcoides.* Different sampling locations within the Houston Ship Channel and a control site as well as different sediment depths were characterized to determine the impact of dioxin-respiring bacteria on total bacterial diversity.

> *Hypothesis 2:* I expect the bacterial diversity in the HSC sediments to be skewed towards toxin-degrading bacteria, such as *Dehalococcoides.*

I tested this hypothesis by constructing 16S rRNA gene clone libraries to determine bacterial diversity. Community DNA was extracted from sediment samples and PCR was performed on the extracted DNA. Carefully chosen samples were cloned and analyzed with restriction enzyme digests. Clones were sequenced to determine bacterial diversity as well as diversity within dioxin-degrading bacteria.

Objective 3

Determine whether or not there were any correlations between the presence of dioxin-degrading bacteria and geochemical and/or sedimentary processes. Since this thesis research was part of a larger effort to better understand dioxin degradation rates and biogeochemical processes in the HSC sediments, my molecular data was analyzed

together with measurements of trace metal concentrations (iron (Fe) and manganese (Mn), nutrient concentrations in pore waters, and radiodating of sediment layers.

Hypothesis 3: I expect to find dechlorinating bacteria only in reducing environments.

I tested this hypothesis by comparing the presence or absence of *Dehalococcoides* to geochemical and sedimentary data. It should be noted that geochemical and radiochemical data for the HSC might be more difficult to interpret due to dredging activities and ship traffic.

Objective 4

I determined the active members of the bacterial community by constructing 16S rRNA gene clone libraries from both DNA and RNA samples. To differentiate the most active fraction(s) of the microbial community from those bacteria which are present but not growing, total community RNA was reverse transcribed into complementary DNA (cDNA) and then amplified with PCR and bacterial specific primers. Since RNA is a lot less stable than DNA, differences in diversity of the clone libraries indicated which groups were active and growing versus the ones which are not.

Hypothesis 4: I expect dechlorinating bacteria to be among the active members of the bacterial community.

I tested this hypothesis by constructing 16S rRNA gene clone libraries from both RNA and DNA. Total RNA and DNA was extracted from the same sample and 16S rRNA gene clone libraries were constructed and analyzed with restriction enzyme digests. Clones were sequenced to determine bacterial diversity. Differences in diversity of these clone libraries was examined to determine the active members of the bacterial community.

I. 2. Materials and Methods

I. 2. 1. Sampling

Sediment cores were collected along the HSC and at a freshwater control site (Fig. 3 and Table 1). Only sediment cores which showed minimal signs of mixing, as determined by X-radiographs, were selected for analysis. Sediment cores were collected as previously described by Yeager et al (64). Each core was sectioned at 1cm intervals over the upper ~50cm and at 2cm intervals thereafter. Sterile technique was applied to every extent possible. Aliquots were collected with an ethanol-flamed spatula, transferred into 50ml sterile Falcon tubes, and frozen at -20°C until later analysis. Shorter sediment cores (~30cm) were collected for RNA analysis at selected sites. RNA sediment cores were sectioned at 1cm intervals as well and aliquots were collected in the same manner as described above. Aliquots were taken within 24 hours of sediment core collection and stored at -80°C until later analysis. Furthermore sediment grab samples were collected from various Texas Bays (Fig. 4) to screen for the presence or absence of *Dehalococcoides* in other Texas Bay systems.

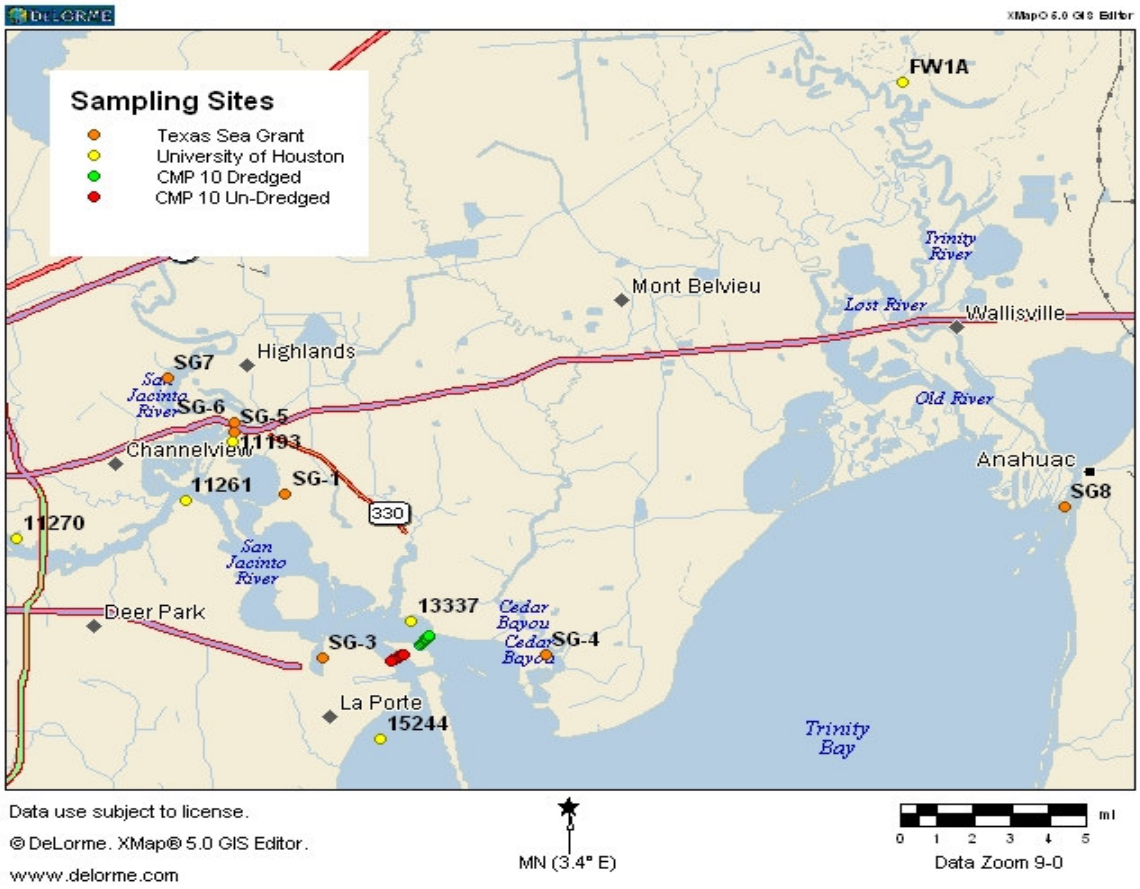

FIG 3. Sampling stations along the Houston Ship Channel and the freshwater control site.

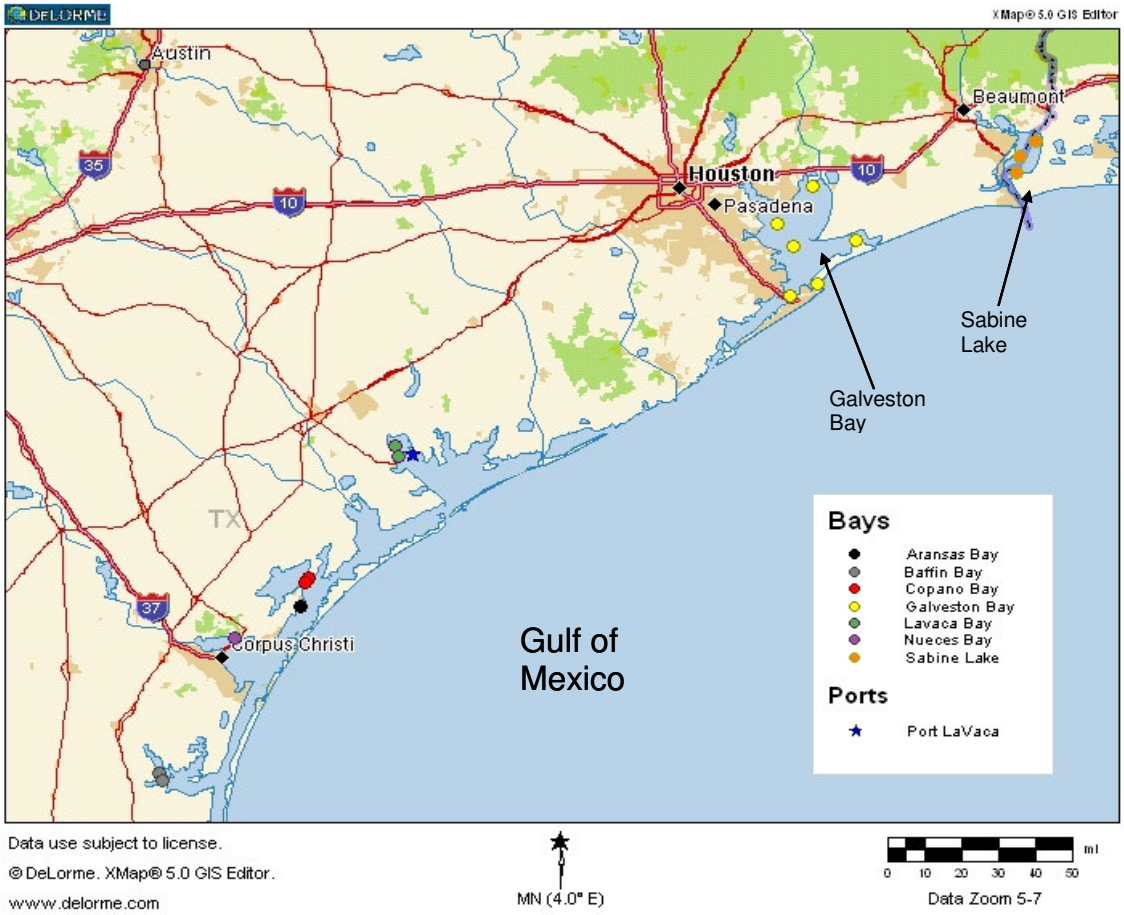

FIG 4. Texas Bays.

TABLE 1. Station coordinates and collection dates.

Station	Latitude	Longitude	Date Collected
SG-1	29° 45' 47.8476" N	95° 2' 22.3908" W	9/29/2006
SG-3	29° 41' 28.8024" N	95° 1' 22.8000" W	9/29/2006
SG-4	29° 41' 34.6200" N	94° 55' 25.0000" W	9/30/2006
SG-6	29° 47' 38.6375" N	95° 3' 43.3800" W	4/14/2007
SG-7	29° 48' 49.5000" N	95° 5' 28.5612" W	4/14/2007
SG-8	29° 45' 25.7976" N	94° 41' 37.7988" W	4/15/2007
11193	29° 47' 8.1599" N	95° 3' 45.0000" W	8/30/2004
11270	29° 44' 36.9959" N	95° 9' 29.7720" W	10/18/2004
11261	29° 45' 36.3600" N	95° 4' 59.1600" W	6/16/2004
15244	29° 39' 21.9601" N	94° 59' 49.5599" W	8/04/2004
13337	29° 42' 28.4760" N	94° 59' 1.2839" W	2/17/2005
FW1A	29° 56' 33.0000" N	94° 45' 57.9600" W	12/10/2004

I. 2. 2. Extraction of Nucleic Acids

Nucleic acids from environmental sediment samples were extracted using either the protocol previously described by Zhou et al (66) or a commercial kit, whichever yielded better results. The Zhou et al (66) protocol uses the enzyme Proteinase K to lyse the bacterial cells. It also uses sodium dodecyl sulfate (SDS) and an extraction buffer, containing Tris, EDTA, sodium chloride, and CTAB, to extract DNA from sediments. The protocol uses chloroform isoamyl alcohol to further extract the DNA and remove contaminants such as organics. Nucleic acids are precipitated with isopropanol. After extraction, the DNA is stored at -20°C for later PCR analysis. Commercial DNA extraction kits we tested include Power Max Soil (MoBio Inc., Solana, CA), Power Soil (MoBio), and Ultra Clean Soil (MoBio).

DNA concentrations were measured with a spectrophotometer since DNA absorbs UV light with an absorption peak at 260nm wavelength. DNA purity can also be measured using a spectrophotometer. Proteins tend to absorb at 280nm and a 260:280 ratio between 1.7 and 2.0 is considered ‘pure’ DNA. Absorption at 230nm is caused by organic compounds and a 260:230 ratio around 2 indicates no organic contamination in the DNA sample. Cell lysis efficiency was determined by microscopic examination of sediment smears before and after extraction. If necessary, nucleic acids were cleaned with a kit such as the WIZARD DNA Cleanup System (Promega Corp., Madison, Wis.).

I. 2. 3. Amplification of the 16S rRNA gene, and Clone Library Construction.

Bacterial diversity was characterized using 16S rRNA gene sequences. The 16S rRNA gene was chosen to characterize bacterial diversity because it is conserved among all cellular life forms and it has a low rate of evolutionary change (13). Within the 16S rRNA gene there are conserved regions, found in all organisms and variable regions, specific to groups or even individual species (13). Due to these characteristics, the 16S rRNA gene trees are used most often for phylogenetic comparisons (17, 19).

The 16S rRNA gene sequences were amplified from the environmental sediment sample nucleic acid extracts by PCR (polymerase chain reaction) with an automated thermal cycler (Eppendorf, Hamburg, Germany) by using the bacterial-specific primers 8*f* and 1492*r* (Table 2 and Fig. 5). PCR is a method which rapidly produces numerous copies of a desired DNA fragment. PCR products were purified with the WIZARD PCR Preps DNA Purification System (Promega). Using a more specific primer set (DET730f and 1492r) (See Table 2) under the conditions previously described by Breitenstein et al (10), a nested PCR was performed on the initial cleaned PCR product to confirm the presence or absence of the bacterial group *Dehalococcoides*.

TABLE 2. Primer sequences.

Primer	**Target**	**Primer Sequence**
8 f	All bacteria	5'-AGA GTT TGA TCC TGG CTC AG-3'
1492 r	All bacteria	5'-TAC GGY TAC CTT GTT ACG ACT T-3'
DET 730 f	Dioxin-respiring bacteria	5'- GCG GTT TTC TAG GTT GTC-3'
DET 1350 r	Dioxin-respiring bacteria	5'- CAC CTT GCT GAT ATG CGG-3'

16S rRNA gene clone libraries were constructed with the pGEM-T-Easy vector system (Promega). Clone libraries allow for the separation of community DNA. The PCR product contains copies of the same DNA fragment (16S rRNA gene) from every organism present. In order to determine the individual species present, each DNA fragment has to be separated. The DNA fragment is ligated or inserted into a circular plasmid, called a vector (ligation reaction). This vector is then inserted into a bacterial cell, usually *E.coli* (transformation reaction). The bacterial cell is then cultured and the resulting colony, which now contains many copies of the desired DNA fragment, is harvested and the circular plasmids are extracted (12) (Fig. 6). The extracted plasmids containing the desired DNA fragment were stored at -20°C for later analysis.

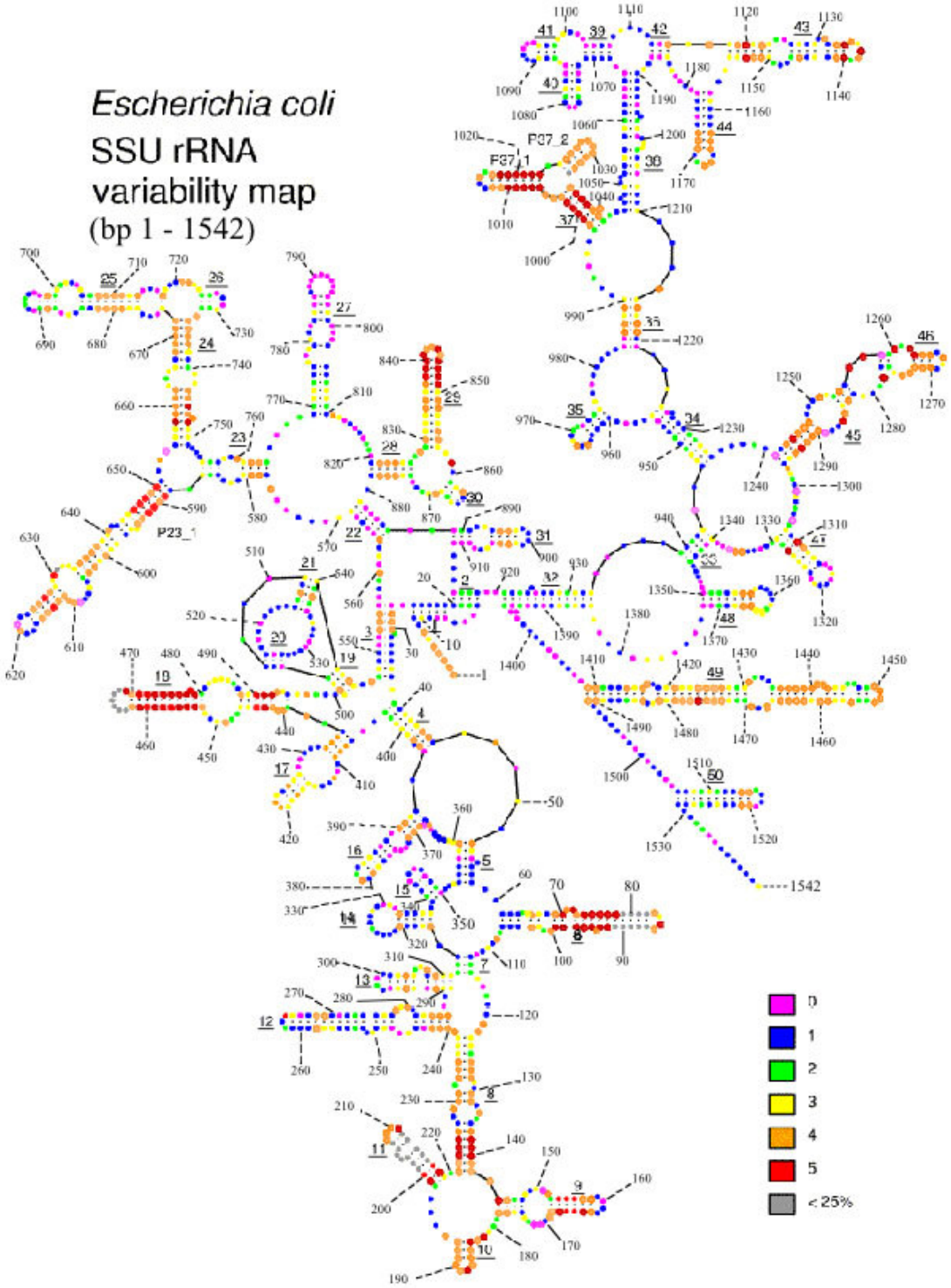

FIG. 5. Map of *Escherichia coli* small 16S rRNA gene (Adapted from Van de Peer, 1996).

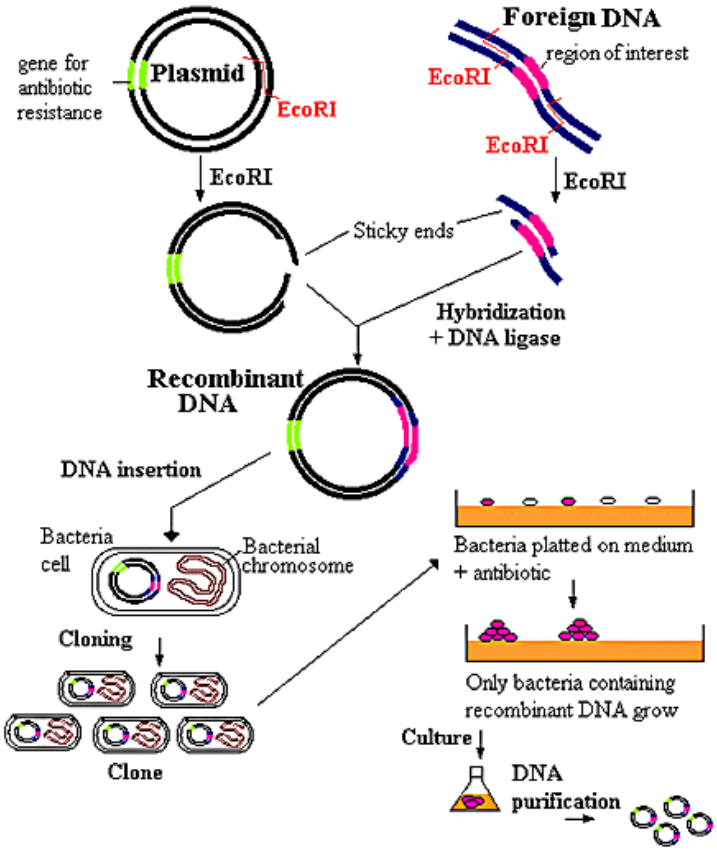

FIG 6. Cloning diagram (www.bio.davidson.edu).

I. 2. 4. ARDRA

Each individual clone was subjected to amplified ribosomal DNA restriction analysis (ARDRA) in order to characterize the 16S rRNA gene diversity within each clone library (32, 59). ARDRA is a genetic fingerprinting technique that is performed on PCR-generated rDNA fragments (61). The PCR products are cut into restriction fragments using endonucleases also called restriction enzymes. Endonucleases only cut the DNA fragments at specific restriction sites, hence creating unique patterns for different bacteria. For this study we used two restriction enzymes, Rsa I and Hae III (Promega).

The restriction enzyme HaeIII was chosen because it has a high average number of restriction sites per taxon (37). However, using only one high-frequency-cutting restriction enzyme may not be very informative phylogenetically. In order to increase phylogenetic resolution we chose RsaI as our second restriction enzyme. RsaI is a low-frequency-cutting restriction enzyme and Moyer et al (37) have shown that RsaI accurately differentiates among many diverse bacterial taxa. The recognition sequences are 5'…GT↓AC…3' and 5'…GG↓CC…3' for Rsa I and Hae III respectively. The resulting ARDRA patterns were separated on an 8% acrylamide gel [19:1, acrylamide / bis-acrylamide]. The gels were stained with ethidium bromide and visualized under UV light. ARDRA patterns were analyzed using the GelCompar software program (Applied Maths, Inc., Austin, TX). The cluster analysis method used was the comparative numerical analysis with the unweighted pair group method using arithmetic averages (UPGMA). Based on this cluster analysis one or in some cases several representatives of each ARDRA pattern group from all clone libraries were selected for sequencing.

I. 2. 5. Sequencing and Phylogenetic Analysis

Sequencing was performed at the DNA Analysis Facility on Science Hill at Yale University. This facility uses dideoxynucleotide sequencing, also called Sanger sequencing or chain-termination sequencing. This type of sequencing is commonly used and utilizes dideoxynucleotides to terminate DNA strand extension, which results in DNA fragments of varying length (50). Sequence data was analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). This software is

commonly used when analyzing 16S rRNA gene diversity. Dendrograms were reconstructed for the phylogenetic analysis. The frequencies of 16S rRNA gene phylotypes determined by ARDRA and subsequent sequencing (i.e., those sharing >97% identity) were used for analysis of diversity. Shannon's index for diversity (H') was calculated according to the method of Zar (65). Shannon's index for diversity is by far the most commonly used diversity index. It takes into account the number of species but also the abundance of each species. Rarefaction curves were interpolated with the freeware program Analytic Rarefaction 1.3 (http://www.uga.edu/_strata/software/index.html). Rarefaction allows one to calculate species richness for a given number of sampled individuals. Rarefaction curves show the number of species as a function of the number of individuals sampled. Hence, a steep slope indicates that a fraction of the species diversity has not been sampled whereas a flattening slope indicates that diversity has been sampled well. Coverage of the clone libraries was estimated as described by Mullins et al. (39). Coverage was derived from the equation

$$C = 1 - (n_1/N)$$

where, n_1 is the number of clones that occurred only once and N is the total number of clones examined. This value is conservative, but excludes variation introduced by PCR artifacts and heterogeneities in rDNA gene families (39).

I. 2. 6. RNA/DNA Extraction

Total RNA and DNA from environmental sediment samples were extracted using the RNA Power Soil Total RNA Isolation Kit (MoBio). Nucleic acids were extracted according to the manufacturer's protocol. Best RNA laboratory technique was applied to every extent possible. Following nucleic acid extraction, the RNA samples were treated with RQ1 DNase (Promega) to degrade any DNA that may have been carried over during the RNA extraction. After the DNase digestion, a phenol-chloroform-isoamyl alcohol extraction followed by an ethanol precipitation was performed as described in Sambrook et al (49). Regular PCR using the bacterial-specific primers 8*f* and 1492*r* (targeting the 16S rRNA gene) was performed on the DNA samples. Reverse transcriptase PCR was performed on the RNA samples using the same bacterial-specific primers 8*f* and 1492*r*. RT-PCR was performed with the Access-Quick RT-PCR system (Promega). In the first step of reverse transcriptase PCR complementary DNA (cDNA) is made from a RNA template. After the cDNA has been generated, a standard PCR reaction follows. Both the DNA and cDNA PCR products were aliquoted and stored at -80°C for later analysis.

I. 2. 7. RNA/DNA Clone Libraries

16S rRNA gene clone libraries were constructed from both DNA and cDNA samples. The same method as described above was used to construct the clone libraries. ARDRA was performed on the clones to determine 16S rRNA gene diversity. Ribosomal RNA is less stable than DNA and should be present in larger quantities in growing cells versus

non-growing cells. It has been shown that active cells have a higher rRNA content than cells that are not active (47). Ravenschlag et al. (47) were able to correlate high sulfate-reducing rates with high cellular rRNA content, indicating that the active sulfate reducers had a higher rRNA content. Using this correlation, I compared the clone libraries originating from DNA and RNA to determine the active members of the bacterial community.

CHAPTER II

SURVEY OF DEHALORESPIRING BACTERIA IN SEDIMENTS OF THE HOUSTON SHIP CHANNEL

II. 1. Introduction

II. 1. 1. Houston Ship Channel

The Houston Ship Channel (HSC), located in the San Jacinto River Basin, in the northwest corner of Galveston Bay, Texas, is 50 miles in length, extending from the Port of Houston to the Gulf of Mexico (Ch. I; Fig. 1). The Port of Houston is the sixth largest seaport in the world and handles more foreign water-borne tonnage than any other U.S. port. The Port of Houston generates over $10 billion annually and each year more than 6,300 vessels pass through the HSC. The HSC is also home to the largest petrochemical complex in the United States and the second largest worldwide (43). The HSC is continually being dredged and the dredged sediment is used to create spoil islands and wetlands. Both the HSC and upper Galveston Bay (GB) are highly polluted with dioxins, dioxin-like compounds, and many other contaminants, such as hydrocarbons, from industrial and municipal effluents and runoff, as well as from atmospheric wet and dry deposition. In 1990, dioxins were detected in fish and crab tissue obtained from the HSC. A seafood consumption advisory for catfish and blue crabs was issued for the HSC and upper GB, and remains in effect to this day. Subsequently, the HSC was placed on the §303 (d) list of impaired water bodies as required by the 1977 Clean Water Act (as amended, 1996) and a total maximum daily loads (TMDL) study was initiated by the

Texas Commission on Environmental Quality (TCEQ). The study revealed that Toxic Equivalent (TEQ) concentrations in water ranged from 0.10 to 3.16 pg TEQ/L and in bottom sediments from 0.9 to 139.8 ng/kg dry wt. (57). On average, dioxin concentrations exceeded the Texas Surface Water Quality Standard (0.093 pg/L) in more than 80% of all samples (48). The study also revealed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the major contributor to total TEQs in all samples. The entire HSC is contaminated with dioxins and recent dioxin inputs to the HSC continue despite regulatory efforts (64).

II. 1. 2. Dioxins

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (dioxins) are persistent environmental contaminants. Dioxins cause a variety of biochemical, immunological, and reproductive effects in animals and are suspected carcinogens (6, 7, 28, 42, 46, 51). Dioxins bioaccumulate in the aquatic and terrestrial food chains posing significant and persistent risks to human health. The estimated half-life of dioxin in the human body is 7-8 years (58). Primary sources of dioxins include the production of herbicides (56), paper and pulp bleaching, metal smelting, and waste incineration (16, 18, 54, 62). Dioxins and dioxin-like compounds are hydrophobic and therefore have a high particle and lipid affinity. Their water solubility is estimated to be 19.3 ng/L (58). Due to their high hydrophobicity, dioxins present in the water column rapidly partition to organic carbon fractions (i.e. black carbon) in suspended soils and can subsequently be buried in sediments (11, 38, 52). Re-suspension of polluted

sediments may re-introduce dioxins into the aquatic food chain; however, this process has not been thoroughly investigated. From both fiscal and environmental perspectives, in situ microbial remediation of dioxins in the HSC and GB is preferable to alternatives, such as removal of contaminated sediments to landfills or chemical treatments. Microbial remediation would also not interfere with the vessel traffic through the HSC. Since the HSC is tidally influenced, dioxin contamination has been transferred up- and downstream of the channel, increasing the urgency of remediation.

II. 1. 3. Microbial Dechlorination

Studies of microbial dechlorination of polychlorinated compounds have been mostly limited to freshwater systems and have indicated that degradation rates are enhanced under anaerobic, reducing conditions (1-Adriaens and Grbic-Galic, 1994). Quensen et al. (44) showed that the chlorinated compound DDE (1,1-dichloro-2,2,-bis(p-chlorophenyl)ethylene), a commercial by-product in DDT formulations, is preferentially degraded under methanogenic and sulfidogenic conditions. Another study found that 2,3,7,8-TCDD was degraded up to 86% under anaerobic, reducing conditions (25) (Ch. I; Fig. 2). The reductive dehalogenation of chlorinated aromatic compounds has been identified as an energy-yielding process in a number of anaerobic bacteria (22). These anaerobic bacteria use polychlorinated compounds as electron acceptors and hydrogen as an electron donor (2, 14, 22). The reductively dechlorinating bacteria known to date belong to the low GC Gram-positive bacteria (*Desulfitobacterium* and *Dehalobacter*) or to the Proteobacteria (for example, *Desulfomonile*, *Desulfuromonas* and

Dehalospirillum) (22, 33). Another bacterial group, *Dehalococcoides*, is also known to reductively dechlorinate highly chlorinated compounds, making the resulting congeners and other biproducts more susceptible to degradation by other bacterial groups. The closest phylogenetic affiliation of *Dehalococcoides* is with the green non-sulfur bacteria (20, 24, 60), however there is increasing evidence that they may constitute a new division of bacteria (22, 33). Thus far, *Dehalococcoides* have only been isolated from groundwater and other freshwater systems. *Dehalococcoides ethenogenes* strain 195, isolated from contaminated groundwater, is the only known isolated organism capable of fully dechlorinating tetrachloroethene (PCE) and other chloroethenes to the non-toxic end-product ethene (2). Strains FL2 (isolated from a highly enriched PCE-to-ethene dechlorinating mixed culture from Red Cedar River sediment, Michigan, Loeffler et al, 2000) and DCEH2 (isolated from a dechlorinating enrichment mixed culture, GenBank accession number AJ249262) also dechlorinate chloroethenes (20). Strain CBDB1 (isolated from an enriched chlorobenzene-dechlorinating mixed culture from Saale River sediment, Germany) dechlorinates trichlorobenzenes and tetrachlorobenzenes to dichlorobenzenes, but is unable to dechlorinate PCE or trichloroethene (2). Strain CBDB1 is also able to dechlorinate chlorinated benzenes (14). Members of *Dehalococcoides* have also been shown to dechlorinate commercial polychlorinated biphenyls (PCBs) (i.e Aroclor 1260) (9). Bedard (9) also found that *Dehalococcoides* obtain energy for growth from dechlorination. In 2003, Bunge et al (14) showed that strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD).

Since the dehalogenation of dioxin by *Dehalococcoides* is orders of magnitude faster (weeks versus 1 to 4 years) than its anaerobic co-metabolic reduction (1, 14) its presence in or addition to dioxin contaminated areas is a significant contribution for bioremediation (1, 2, 14, 22).

II. 1. 4. Objective

I screened contaminated sediments from the HSC for the presence or absence of the bacterial group *Dehalococcoides,* since it is known to reductively dehalogenate dioxins and other chlorinated compounds such as chloroethenes and chlorobenzenes. *Dehalococcoides* are substrate specific bacteria and use dioxins and similar compounds exclusively as their energy source. This characteristic makes them a good indicator for dioxin contamination in the sediment. Moreover, this assessment of the geographic and vertical distribution of *Dehalococcoides* in HSC sediments is important for estimating the potential for accelerated bioremediation of dioxins.

> *Hypothesis 1:* I expect to find the bacterial group *Dehalococcoides* at higher dioxin concentrations in the sediments, but not at lower to zero concentrations.

The hypothesis was tested by comparing the presence or absence of *Dehalococcoides* to dioxin concentrations. I extracted community DNA from sediment samples and performed PCR, first with general bacterial primers and then with *Dehalococcoides*-specific primers. PCR products were visualized on agarose gels stained with ethidium

bromide. Dioxin concentrations were also measured for selected sediment samples and these results were compared to the PCR results.

II. 2. Materials and Methods

II. 2. 1. Sampling

Sediment cores were collected along the HSC and at a freshwater control site (Ch. I, Fig. 3 and Table 1). Cores were collected for two projects. Cores collected for the first project conducted by the University of Houston (UH) and TAMUG (2003-2005) are named according to Texas Commission on Environmental Quality (TCEQ) water quality sampling sites for the HSC and have six numerical characters. Cores collected for the second project (funded by the Texas Sea Grant College) have 2 letter characters (SG) followed by the station number (1, 2, 3, etc). Only sediment cores which showed minimal signs of mixing, as determined by X-radiographs, were selected for analysis. Sediment cores were collected as previously described by Yeager et al (64). Each core was sectioned at 1cm intervals over the upper ~50cm and at 2cm intervals thereafter. Sterile technique was applied to every extent possible. Aliquots were collected with an ethanol-flamed spatula, transferred into 50ml sterile conical tubes, and frozen at -20°C until later analysis. Furthermore sediment grab samples were collected from several Texas Bays (Ch. I, Fig. 4) to screen for the presence or absence of *Dehalococcoides* in other Texas Bay systems.

II. 2. 2. Extraction of Nucleic Acids

Nucleic acids from environmental sediment samples were extracted using either the protocol previously described by Zhou et al (66) or a commercial kit, whichever yielded better results. The Zhou et al (66) protocol was slightly modified for our experiments. Five grams of frozen sediment were weighed in a 50ml sterile conical centrifuge tube. Thirteen and one half ml extraction buffer and 100µl (10mg/ml) of the enzyme Proteinase K were added to the sediment. The enzyme Proteinase K is often used to lyse bacterial cells. The 50 ml conical tubes were placed in a shaking (240 rpm) 37°C water bath and incubated for 30 minutes. After the shaking water bath, 1.5ml 20% sodium dodecyl sulfate (SDS) was added and the tubes were put in a 65°C water bath for 2 hours. After the 2 hour incubation period, the sediment was centrifuged at 6,500 × g, for 10 minutes at room temperature (20°C). The supernatant was transferred into a new 50 ml conical tube with a sterile Pasteur pipet. The sediment pellet was extracted two more times by adding 4.5ml extraction buffer and 0.5ml 20% SDS. The tubes were briefly vortexed to loosen the pellet and incubated at 65°C for 10 minutes. The tubes were centrifuged as before and the supernatant was combined. Following the pellet extraction was a chloroform cleaning step. An equal volume of chloroform isoamyl alcohol (24:1) was added to the supernatant. The tubes were inverted until an emulsion formed and centrifuged at 6,500 × g for 15 minutes at room temperature (20°C). The upper aqueous phase was removed with a Pasteur pipet and transferred into a new sterile 50ml Falcon tube. This was followed by isopropanol precipitation. 0.6 volume of cold

(-20°C) 100% isopropanol was added to the aqueous phase. The tubes were inverted and the DNA was allowed to precipitate in the freezer overnight. The next day the tubes were centrifuged at 6,500 × g for 1 hour at 4°C. The supernatant was decanted and the DNA pellet washed with 80% ice-cold ethanol to remove any remaining salts. About 1ml of ice-cold 80% ethanol was added to the pellet and the tubes were centrifuged again for 5 minutes (6,500 × g and 4°C). The DNA pellets were allowed to air dry overnight. The next day they were resuspended in 500µl LT buffer and transferred to 2ml Eppendorf tubes and centrifuged at 6,500 × g for 15 minutes at 4°C. The supernatant was transferred to a new 2ml tube and stored at -20°C for later PCR analysis.

The following commercial DNA extraction kits were also used to determine if they could successfully extract community DNA from HSC sediments: Power Max Soil (MoBio Inc., Solana, CA), Power Soil (MoBio), and Ultra Clean Soil (MoBio). Cell lysis efficiency was determined by microscopic examination of sediment smears before and after extraction. Due to time-constraints, this was only done in the beginning of the study to verify the extraction method efficiency.

Extracted nucleic acids required an additional clean up step with the WIZARD DNA Cleanup System (Promega Corp., Madison, Wis.) conducted according to the manufacturer's protocol. In most cases, the eluate from the first minicolumn was purified further with fresh resin and passage through another minicolumn. This second

purification step was necessary to remove PCR inhibitors that were co-extracted from the samples. These added cleaning steps did not always remove PCR inhibitors. The DNA concentrations and the 260/280 absorbance ratios were recorded for each DNA sample after extraction.

II. 2. 3. PCR Amplification

16S rRNA gene sequences were amplified from the environmental sediment sample nucleic acid extracts by PCR with an automated thermal cycler (Eppendorf, Hamburg, Germany) by using the bacterium-specific primers 8*f* (*5'-agagtttgatcctggctcag-3'*) and 1492*r* (*5'-tacggytaccttgttacgactt-3'*). The PCR reactions were 50µl in volume and contained 0.5µl of each primer (10µmol), 1µl dNTPs (10mmol), 1µl BSA, 5µl PCR buffer (Roche, Basel, Switzerland), 0.5µl Taq (Roche). Final volume was reached with DNAase/RNAase-free PCR water. From 1µl to 5µl DNA template (100 ng/ µl) was added. PCR conditions were as follows: initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 3 minutes, followed by a final extension at 72°C for 10 minutes. 10µl of the PCR products were run on a 1% agarose gel at 100V, stained with ethidium bromide, and visualized under UV light (Fig. 7, PCR with bacterium-specific primers).

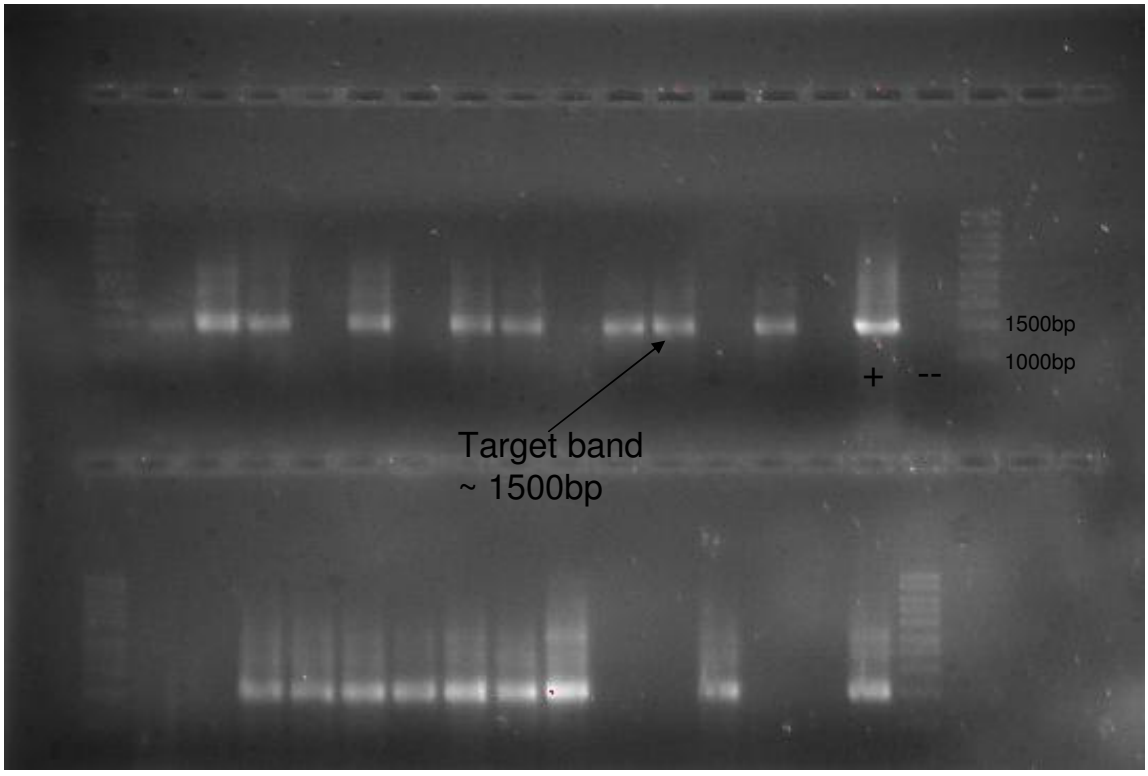

FIG 7. 1% agarose gel image showing the PCR results of samples amplified with bacterium-specific primers. Positive control (+) was *E.coli* and negative (--) control was no DNA.

PCR products were purified with the WIZARD PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol. Using a more specific primer set (DET730f (*5'-gcggttttctaggttgtc-3'*)and 1492r) that targets *Dehalococcoides* under the conditions previously described by Breitenstein et al (10), a nested PCR was performed on the initial PCR product to confirm the presence or absence of the bacterial group *Dehalococcoides*. PCR reactions were the same as described above. (Fig. 8, PCR with Dehaloccoides specific primers).

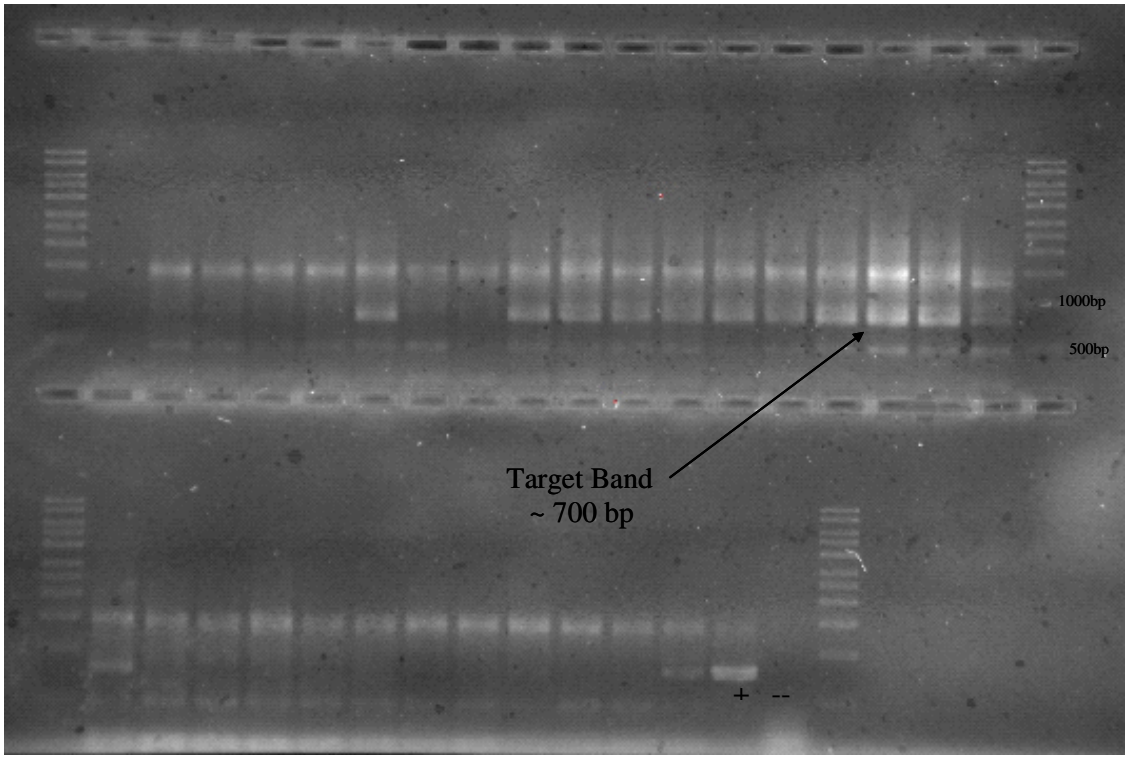

FIG 8. 1% agarose gel image showing the PCR results of samples amplified with *Dehalococcoides*-specific primers. Positive control (+) was *Dehalococcoides* CBDB1 and negative (--) control was *E.coli*.

II. 3. Results and Discussion

II. 3. 1. Results

Tables 3-? show the results for all the cores examined. The sedimentary data for cores 11193, 11270, 15244, 11261, 13337, and FW1A were taken from Yeager et al (64). Dr. Kevin Yeager is a co-Principal Investigator on the UH/TAMUG project and the TAMUG-Sea Grant project. These data include dioxin concentrations, estimated sediment ages, and POC (particulate organic carbon). POC (from sediment) for cores SG 1, 3, 4, 6, 7, and 8 was analyzed by Chen Xu, a PhD candidate in Dr. Peter Santschi's laboratory, using a CHN analyzer. Dioxin concentrations for cores SG 1, 3, and 4 were

determined by Dr. Ying Hung Shen in Taiwan. Dr. Yeager's lab at the University of Southern Mississippi conducted the radiochemical analyses for estimation of sediment deposition rates (i.e. age of sediment layers).

Station 11193

This station showed little mixing in the short term. It had the second highest dioxin concentrations out of all the UH/TAMUG cores (11193, 11270, 15244, 11261, 13337, and FW1A). *Dehalococcoides* was first detected at 6cm (about 1.5 years old sediment) and detected in all sediment depths analyzed thereafter except 22cm. No PCR product for certain depths indicates PCR inhibition (Table 3).

TABLE 3. Station 11193.

Station 11193 (N29.7856°, W95.0625°)							
Depth (cm)	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	< 1	16.00	40.00	22.20	4.70 ± 0.02
2	+	-	--	12.00	34.00	17.55	4.23 ± 0.02
3	+	-	--	6.40	16.00	8.72	4.13 ± 0.02
4	+	-	--	10.00	28.00	14.82	4.15 ± 0.02
5	+	-	--	6.00	17.00	8.71	3.20 ± 0.01
6	+	+	--	--	--	--	4.45 ± 0.03
7	+	+	--	--	--	--	4.65 ± 0.03
8	+	-	--	9.40	23.00	13.79	4.37 ± 0.03
9	I[4]	?	--	--	--	--	4.53 ± 0.03
10	I	?	--	10.00	24.00	15.21	3.60 ± 0.03
11	I	?	--	--	--	--	3.70 ± 0.03
12	I	?	--	--	--	--	2.75 ± 0.02
13	+	+	--	--	--	--	2.80 ± 0.02
14	+	+	--	--	--	--	3.30 ± 0.02
15	+	+	--	5.10	12.00	7.28	3.10 ± 0.02
16	I	?	2.49	--	--	--	3.90 ± 0.03
17	+	+	3.98	--	--	--	3.45 ± 0.03
18	I	?	5.47	--	--	--	4.45 ± 0.03
19	I	?	6.96	--	--	--	3.95 ± 0.03
20	+	+	8.45	6.80	17.00	9.71	3.43 ± 0.03
21	+	+	9.94	--	--	--	2.85 ± 0.03
22	+	-	11.43	--	--	--	3.25 ± 0.03
23	I	?	12.92	--	--	--	3.25 ± 0.03
24	I	?	14.41	--	--	--	3.33 ± 0.03
27	I	?	18.88	--	--	--	2.65 ± 0.02
31	+	+	24.84	5.90	14.00	8.58	--
40	+	+	38.25	11.00	18.00	13.79	--

[1] PCR product with universal bacterial primers 8f and 1492r.

[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.

[3] + denotes positive PCR product, - denotes negative PCR product.

[4] No PCR product indicating reaction inhibition.

Station 11270

This sediment core showed episodic, rapid sedimentation. It had the highest dioxin concentrations out of all the TCEQ cores. It had less than average annual present-day dioxin fluxes compared to all the other TCEQ sites. It appears this site has been affected by high-energy depositional events (64). *Dehalococcoides* was first detected at 3cm (about 3.48 years old) and it was detected in all sediment depths analyzed thereafter. No PCR product for certain depths again indicates PCR inhibition (Table 4).

TABLE 4. Station 11270

11270 (N29.74361°, W95.15827°)							
Depth (cm)	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	I[4]	?	1.16	--	--	--	5.27 ± 0.02
2	I	?	2.32	1.60	3.90	2.41	5.70 ± 0.02
3	+[3]	+	3.48	--	--	--	9.33 ± 0.03
4	+	+	4.64	26.00	57.00	36.29	6.60 ± 0.02
5	+	+	5.80	--	--	--	5.85 ± 0.02
8	+	+	9.28	48.00	96.00	59.54	3.15 ± 0.01
12	+	+	13.92	190.00	420.00	238.95	4.90 ± 0.01
16	+	+	18.56	100.00	230.00	128.51	6.90 ± 0.03
20	+	+	23.20	96.00	230.00	128.45	7.30 ± 0.03
24	I	?	27.84	59.00	140.00	80.50	9.05 ± 0.04
28	I	?	32.48	120.00	330.00	171.50	8.18 ± 0.04
32	+	+	37.12	85.00	250.00	137.35	--
37	I	?	42.92	89.00	180.00	112.38	--
41	+	+	47.56	1.50	3.50	2.09	--
43	+	+	49.88	3.20	6.00	4.21	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Station 15244

This sediment core displayed a stable sedimentary setting, with mixing confined to the near surface (0-2cm). *Dehalococcoides* was first detected at 8cm (about 7.12 years old). It was not detected at 10cm and 15 cm but was again detected at 30cm (about 34.35 years old). Again, some depths did not produce a PCR product (Table 5).

TABLE 5. Station 15244.

Station 15244 (N29.6561°, W94.9971°)							
Depth	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1cm	+[3]	-	0.78	3.30	8.00	6.76	7.13 ± 0.03
2cm	+	-	1.66	--	--	--	7.20 ± 0.03
3cm	+	-	2.45	4.60	12.00	9.26	5.50 ± 0.02
4cm	I[4]	?	3.31	4.50	12.00	8.55	3.85 ± 0.01
5cm	+	-	4.21	2.30	6.00	4.85	4.80 ± 0.02
6cm	I	?	5.37	3.00	7.20	5.71	4.03 ± 0.02
7cm	+	-	6.27	--	--	--	3.87 ± 0.02
8cm	+	+	7.12	--	--	--	3.70 ± 0.01
9cm	I	?	8.09	--	--	--	4.70 ± 0.02
10cm	+	-	9.16	2.30	5.60	4.48	3.80 ± 0.01
15cm	+	-	14.96	1.60	3.60	3.10	4.40 ± 0.03
21cm	I	?	22.16	0.84	2.90	2.27	4.60 ± 0.01
30cm	+	+	34.35	1.90	3.90	3.64	4.55 ± 0.01
40cm	I	?	53.84	1.10	2.30	1.74	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Station 11261

This sediment core showed rapid sedimentation. It had less than average dioxin concentrations for all TCEQ sites (except FW1A). It had the highest sediment accumulation rate as well as the highest present-day dioxin fluxes. *Dehalococcoides* was first detected at 30cm (about 5.2 years old), 40cm had no PCR product indicating PCR inhibition (Table 6).

TABLE 6. Station 11261.

Station 11261 (N29.7601°, W95.0831°)							
Depth (cm)	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	0.02	2.70	7.10	4.15	3.80 ± 0.01
2	+	-	0.04	--	--	--	3.40 ± 0.01
3	+	-	0.06	3.70	9.70	5.64	4.40 ± 0.01
4	+	-	0.08	--	--	--	3.85 ± 0.01
6	+	-	0.12	3.90	11.00	6.15	2.15*
7	+	-	0.14	7.00	20.00	11.16	3.60 ± 0.01
8	+	-	0.16	10.00	28.00	15.80	6.17 ± 0.04
11	+	-	0.22	2.70	7.60	3.10	2.53 ± 0.01
13	+	-	0.26	4.10	13.00	6.33	3.50 ± 0.02
17	+	-	0.34	2.80	7.20	4.24	3.50 ± 0.02
20	+	-	0.40	2.70	6.30	3.96	3.10 ± 0.02
30	+	+	5.20	2.00	5.30	2.98	4.25 ± 0.01
40	I[4]	?	10.0	3.90	8.70	5.76	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Station 13337

This core had little surface mixing in the short term. *Dehalococcoides* was first detected at 11cm (about 6.3 years old). It was detected in all sediment depths analyzed thereafter (except where no PCR product) (Table 7).

TABLE 7. Station 13337.

13337 (N29.70791°, W94.98369°)							
Depth (cm)	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	--	4.60	14.00	9.32	11.30 ± 0.06
2	+	-	--	1.70	5.10	3.30	11.15 ± 0.06
3	+	-	--	4.90	15.00	10.22	9.95 ± 0.05
4	+	-	1.93	4.90	14.00	9.82	9.50 ± 0.05
5	+	-	2.51	4.80	16.00	9.28	9.75 ± 0.05
7	+	-	3.63	--	--	--	8.45 ± 0.05
8	I[4]	?	4.28	--	--	--	8.75 ± 0.05
9	+	-	4.98	--	--	--	8.55 ± 0.05
10	+	-	5.63	--	--	--	9.60 ± 0.05
11	+	+	6.3	3.70	11.00	8.22	9.85 ± 0.06
15	+	+	8.85	3.80	11.00	7.65	9.45 ± 0.05
20	I	?	12.66	3.80	11.00	6.84	8.05 ± 0.05
25	+	+	16.32	3.30	9.10	6.87	8.55 ± 0.05
30	+	+	20.14	7.80	20.00	12.81	8.75 ± 0.05
35	I	?	24.96	8.20	24.00	13.17	--
40	+	+	30.17	7.30	30.00	14.47	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Station FW1A

This station was chosen as a control site (Ch. I; Fig. 3) to measure natural background concentrations of dioxins. This sediment core had short term mixing confined to the near surface (0-2cm). Dioxin was present at very low concentrations, compared to all the other cores. *Dehalococcoides* was not detected at 15cm, 20cm, 25cm, 30cm, and 40cm. These five depths were the only ones where PCR was not inhibited by contaminants. However, it is unlikely that *Dehalococcoides* is present at shallower depths in this core (Table 8).

TABLE 8. Station FW1A.

Wetlands Control Station FW1A (N29.9425°, W94.7661°)							
Depth (cm)	**PCR product for Bacteria [1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	I	?	2.77	0.24	1.2	2	67.58 ± 0.24
2	I	?	4.8	0.16	0.3	0.65	43.55 ± 0.16
3	I	?	7.63	0.19	0.26	0.68	24.25 ± 0.09
4	I	?	11.2	0.17	0.24	0.59	21.90 ± 0.08
5	I	?	15.7	0.16	0.24	0.34	8.75 ± 0.03
6	I	?	--	--	--	--	5.00 ± 0.02
7	I	?	17.3	--	--	--	8.70 ± 0.03
8	I	?	18.51	--	--	--	4.50 ± 0.02
9	I	?	20.22	--	--	--	4.40 ± 0.02
10	I	?	21.38	--	--	--	5.00 ± 0.02
11	I	?	22.91	0.16	0.24	0.32	5.38 ± 0.02
12	I	?	23.75	--	--	--	4.40 ± 0.02
15	+	-	26.55	0.16	0.24	0.28	4.25 ± 0.02
20	+	-	31.29	0.16	0.23	0.35	3.50 ± 0.02
25	+	-	35.49	0.28	0.24	0.42	3.30 ± 0.01
30	+	-	39.9	0.16	0.23	0.27	3.05 ± 0.01
40	+	-	55.54	0.16	0.26	0.32	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Station SG-1

This sediment core showed PCR inhibition at every depth from which nucleic acids were extracted. Multiple cleaning steps and varying DNA concentrations in PCR reactions yielded no results. This core is only shown for completeness and effort (Table 9).

TABLE 9. Station SG-1.

Station SG-1 (N29.763291°, W95.039553°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	I[4]	?	NYA[5]	2.26	23.88	8.655	2.18 ± 0.5
2	I	?	NYA	--	--	--	3.38 ± 0.5
3	I	?	NYA	--	--	--	2.30 ± 0.4
4	I	?	NYA	--	--	--	2.29 ± 0.1
5	I	?	NYA	--	--	--	2.28 ± 0.4
6	I	?	NYA	--	--	--	2.47 ± 0.3
7	I	?	NYA	--	--	--	2.31 ± 0.3
8	I	?	NYA	--	--	--	2.41 ± 0.1
9	I	?	NYA	--	--	--	2.22 ± 0.2
10	I	?	NYA	3.36	5.57	12.47	2.30 ± 0.4
15	I	?	NYA	--	--	--	2.54 ±0.04
20	I	?	NYA	5.00	4.56	19.34	2.23 ± 0.2
25	I	?	NYA	--	--	--	1.38 ± 0.1
30	I	?	NYA	--	--	--	2.10 ± 0.3
35	I	?	NYA	--	--	--	--
40	I	?	NYA	1.31	9.28	9.07	--
45	I	?	NYA	--	--	--	--
50	I	?	NYA	--	--	--	--
60	I	?	NYA	--	--	--	--
70	I	?	NYA	--	--	--	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.
[5] Data are not yet available.

Station SG-3

Dehalococcoides was first detected at 5cm. It was detected at all sediment depths analyzed thereafter, except the ones which had no PCR product (Table 10).

TABLE 10. Station SG-3.

Station SG-3 (N29.691334°, W95.023°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	NYA[5]	2.27	23.88	8.65	1.16 ± 0.3
2	+	-	NYA	--	--	--	1.16 ± 0.3
3	+	-	NYA	--	--	--	1.08 ± 0.2
4	I[4]	?	NYA	--	--	--	1.03 ± 0.2
5	+	+	NYA	--	--	--	1.05 ± 0.2
6	I	?	NYA	--	--	--	0.98 ± 0.1
7	I	?	NYA	--	--	--	0.96 ± 0.1
8	I	?	NYA	--	--	--	0.93 ± 0.1
9	+	+	NYA	--	--	--	0.86 ± 0.1
10	+	+	NYA	3.36	5.57	12.47	0.81 ± 0.1
15	I	?	NYA	--	--	--	0.87 ± 0.01
20	+	+	NYA	5.0	4.56	19.34	0.75 ± 0.1
25	I	?	NYA	--	--	--	0.91 ± 0.1
30	I	?	NYA	--	--	--	0.62 ± 0.03
35	I	?	NYA	--	--	--	--
40	I	?	NYA	1.31	9.28	9.07	--
45	I	?	NYA	--	--	--	--
50	I	?	NYA	--	--	--	--
60	I	?	NYA	--	--	--	--
70	+	-	NYA	--	--	--	1.16 ± 0.3

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.
[5] Data are not yet available.

Station SG-4

Dehalococcoides was first detected at 2cm. It was also detected at 3cm, but not at 4cm and 8cm. It was, however, detected again at 10cm, 30cm, and 40cm. The remaining depths had no PCR products, indicating PCR inhibition (Table 11).

TABLE 11. Station SG-4.

Station SG-4 (N29.69295°, W94.923611°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	NYA[5]	3.88	9.76	7.93	1.16 ± 0.1
2	+	+	NYA	--	--	--	1.49 ± 0.4
3	+	+	NYA	--	--	--	1.61 ± 0.5
4	+	?	NYA	--	--	--	1.60 ± 0.6
5	+	-	NYA	--	--	--	1.58 ± 0.6
6	I[4]	?	NYA	--	--	--	1.62 ± 0.6
7	I	?	NYA	--	--	--	1.60 ± 0.6
8	+	-	NYA	--	--	--	1.51 ± 0.6
9	I	?	NYA	--	--	--	1.58 ± 0.5
10	+	+	NYA	2.09	6.32	8.33	1.43 ± 0.5
15	I	?	NYA	--	--	--	1.48 ± 0.2
20	I	?	NYA	2.70	9.08	10.36	1.49 ± 0.6
25	I	?	NYA	--	--	--	1.76 ± 0.03
30	+	+	NYA	--	--	--	1.46 ± 0.7
35	I	?	NYA	--	--	--	--
40	+	+	NYA	26.93	86.91	13.13	--
50	I	?	NYA	--	--	--	--
60	I	?	NYA	--	--	--	--
70	I	?	NYA	--	--	--	--
80	I	?	NYA	--	--	--	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.
[5] Data are not yet available.

Station SG-6

This sediment core was collected in the so-called 'Dioxin Pit'. At this site, a former paper mill subsided and sediments have been shown to have high dioxin inventories (57). *Dehalococcoides* was first detected in 1cm, but not at 3cm, 4cm, or 5cm. It was detected again from 6cm to 10cm, not at 15cm, and 10 cm horizons from 20 to 70cm showed PCR inhibition (Table 12).

TABLE 12. Station SG-6.

Station SG-6 (N29.794066°, W95.06205°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	+	NYA[5]	NYA	NYA	NYA	1.65 ± 0.6
2	I[4]	?	NYA	NYA	NYA	NYA	2.33 ± 1.23
3	+	-[3]	NYA	NYA	NYA	NYA	2.19 ± 1.17
4	+	-	NYA	NYA	NYA	NYA	1.52 ± 0.4
5	+	-	NYA	NYA	NYA	NYA	2.02 ± 0.5
6	+	+	NYA	NYA	NYA	NYA	1.88 ± 0.8
7	+	+	NYA	NYA	NYA	NYA	1.77 ± 0.6
8	+	+	NYA	NYA	NYA	NYA	2.50 ± 0.8
9	+	+	NYA	NYA	NYA	NYA	2.12 ± 0.8
10	+	+	NYA	NYA	NYA	NYA	2.09 ± 0.9
15	+	-	NYA	NYA	NYA	NYA	1.47 ± 0.1
20	I	?	NYA	NYA	NYA	NYA	1.93 ± 0.3
25	I	?	NYA	NYA	NYA	NYA	1.31 ± 0.2
30	I	?	NYA	NYA	NYA	NYA	3.12 ± 0.2
35	I	?	NYA	NYA	NYA	NYA	--
40	I	?	NYA	NYA	NYA	NYA	--
45	I	?	NYA	NYA	NYA	NYA	--
50	I	?	NYA	NYA	NYA	NYA	--
60	I	?	NYA	NYA	NYA	NYA	--
70	I	?	NYA	NYA	NYA	NYA	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.
[5] Data are not yet available.

Station SG-7

This sediment core was collected in the San Jacinto River to determine if dioxin contamination was transported upstream from 'Dioxin Pit' (Station SG-6). This core showed extensive PCR inhibition and multiple cleanup steps and varying DNA concentrations in PCR reactions yielded no results. *Dehalococcoides* was, however, detected at 3cm. Unfortunately, every depth thereafter showed PCR inhibition (Table 13).

TABLE 13. Station SG-7.

Station SG-7 (N29.81375°, W95.091267°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	NYA[5]	NYA	NYA	NYA	1.73 ± 0.1
2	+	-	NYA	NYA	NYA	NYA	1.70 ± 0.1
3	+	+	NYA	NYA	NYA	NYA	1.81 ± 0.01
4	I[4]	?	NYA	NYA	NYA	NYA	1.95 ± 0.1
5	I	?	NYA	NYA	NYA	NYA	2.15 ± 0
6	I	?	NYA	NYA	NYA	NYA	1.94 ± 0.2
7	I	?	NYA	NYA	NYA	NYA	1.75 ± 0.02
8	I	?	NYA	NYA	NYA	NYA	1.73 ± 0.2
9	I	?	NYA	NYA	NYA	NYA	1.59 ± 0.1
10	I	?	NYA	NYA	NYA	NYA	1.68 ± 0.2
15	I	?	NYA	NYA	NYA	NYA	1.57 ± 0.4
20	I	?	NYA	NYA	NYA	NYA	1.13 ± 0.2
25	I	?	NYA	NYA	NYA	NYA	1.38 ± 0.3
30	I	?	NYA	NYA	NYA	NYA	1.77 ± 0.8
35	I	?	NYA	NYA	NYA	NYA	--
40	I	?	NYA	NYA	NYA	NYA	--
45	I	?	NYA	NYA	NYA	NYA	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.
[5] Data are not yet available.

Station SG-8

This sediment core was collected at a 'pristine' site in north Galveston Bay close to the mouth of the Trinity River and within the Anuhuac National Wildlife Refuge. However, *Dehalococcoides* bacteria were detected in this core. A second PCR was performed with the DET730f and DET1350r *Dehalococcoides*-specific primers and indeed, *Dehalococcoides* was detected again. Although, dioxin values are not yet available for this core, the presence of *Dehalococcoides* is a strong indicator of dioxin contamination. The source of the dioxins (air deposition or other) has yet to be determined (Table 14).

TABLE 14. Station SG-8.

Station SG-8 (N29.757166°, W94.693833°)							
Depth (cm)	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**	**Est. Age (yr)**	**2,3,7,8 TCDD (ng/kg-dry wt)**	**2,3,7,8 TCDF (ng/kg-dry wt)**	**Total TEQ (ng/kg-dry wt)**	**POC (mg/g)**
1	+[3]	-[3]	NYA[5]	NYA	NYA	NYA	1.00 ± 0.2
2	I[4]	?	NYA	NYA	NYA	NYA	1.06 ± 0.03
3	+	-	NYA	NYA	NYA	NYA	0.84 ± 0.03
4	+	+, (+)[6]	NYA	NYA	NYA	NYA	0.85 ± 0.04
5	+	+, (+)	NYA	NYA	NYA	NYA	0.95 ± 0.1
6	+	+, (-)	NYA	NYA	NYA	NYA	1.09 ± 0.4
7	I	?	NYA	NYA	NYA	NYA	2.64 ± 1.8
8	+	+, (-)	NYA	NYA	NYA	NYA	1.06 ± 0.3
9	I	?	NYA	NYA	NYA	NYA	0.95 ± 0.1
10	+	+, (-)	NYA	NYA	NYA	NYA	0.92 ± 0.2
11	+	+, (+)	NYA	NYA	NYA	NYA	1.02 ± 0.2
12	+	+, (-)	NYA	NYA	NYA	NYA	1.02 ± 0.01
13	+	+, (+)	NYA	NYA	NYA	NYA	1.04 ± 0.1
14	+	+, (+)	NYA	NYA	NYA	NYA	1.18 ± 0.03
15	+	+, (-)	NYA	NYA	NYA	NYA	0.98 ± 0.1
20	+	+, (+)	NYA	NYA	NYA	NYA	1.17 ± 0.1
25	+	+, (+)	NYA	NYA	NYA	NYA	0.85 ± 0.1
30	+	+, (+)	NYA	NYA	NYA	NYA	1.26 ± 0.1
35	+	+, (+)	NYA	NYA	NYA	NYA	--
40	+	+	NYA	NYA	NYA	NYA	--

TABLE 14. Continued.

Depth (cm)	PCR product for Bacteria[1]	PCR product for Dehalococcoides[2]	Est. Age (yr)	2,3,7,8 TCDD (ng/kg-dry wt)	2,3,7,8 TCDF (ng/kg-dry wt)	Total TEQ (ng/kg-dry wt)	POC (mg/g)
45	+	+	NYA	NYA	NYA	NYA	--
50	+	+	NYA	NYA	NYA	NYA	--
60	I	?	NYA	NYA	NYA	NYA	--
70	+	+	NYA	NYA	NYA	NYA	--

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3]+ denotes positive PCR product, - denotes negative PCR product.
[4]No PCR product indicating reaction inhibition.
[5]Data are not yet available.
[6]PCR product with *Dehalococcoides*-specific primers DET730f and DET1350r.

CMP-10 Stations: Dredged versus Undredged

We also investigated the impact of dredging upon the presence or absence of *Dehalococcoides. Dehalococcoides* was detected in both the dredged and undredged samples (Table 15)

TABLE 15. CMP-10 stations.

CMP-10					
Location	PCR product for Bacteria[1]	PCR product for Dehalococcoides[2]	2,3,7,8 TCDD (ng/kg-dry wt)	2,3,7,8 TCDF (ng/kg-dry wt)	Total TEQ (ng/kg-dry wt)
D1	+[3]	+	1.14	3.64	4.39
D2	+	+	ND[4]	1.85	1.54
D3	+	+	2.03	5.04	7.02
D4	+	+	ND	6.06	5.24
D5	+	+	1.02	3.15	7.69
D6	+	-[3]	ND	1.38	3.64
D7	+	+	0.63	1.64	5.77
D8	+	+	6.30	32.22	13.31
D9	+	+	11.38	23.88	22.97
UD1	+	+	2.08	4.70	6.38
UD2	+	+	2.07	4.78	6.32

TABLE 15. Continued.

Location	PCR product for Bacteria[1]	PCR product for Dehalococcoides[2]	2,3,7,8 TCDD (ng/kg-dry wt)	2,3,7,8 TCDF (ng/kg-dry wt)	Total TEQ (ng/kg-dry wt)
UD3	+	-	1.75	4.85	5.42
UD4	+	-	2.71	12.12	8.44
UD5	+	+	4.36	10.98	12.51
UD6	+	-	3.60	10.15	12.85
UD7	+	+	3.39	12.02	12.29
UD8	+	-	3.20	10.73	13.72
UD9	+	+	1.89	5.11	6.60

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] Not detectable/below detection limits

Texas Bays

Sediment grab samples were also collected from seven Texas Bays and other water coastal bodies to determine if *Dehalococcoides* was present in other bay systems in addition to Galveston Bay and the HSC (Ch. I, Fig. 4). *Dehalococcoides* was detected in Galveston Bay at stations 4, 5, and 6 located at the mouth of the Trinity River in 'Trinity Bay' and along the ship channel within the main Galveston Bay (Fig. 9). Two grab samples from Sabine Lake also tested positive for *Dehalococcoides* (Fig. 10). *Dehalococcoides* were not detected in any of the other bays or coastal water bodies (Table 16).

TABLE 16. Texas Bays.

Texas Bays			
Location	**Station**	**PCR product for Bacteria[1]**	**PCR product for Dehalococcoides[2]**
Aransas Bay, TX (ARB)	1	I[4]	?
	2	+[3]	-[3]
Baffin Bay, TX (BFB)	1	+	-
	2	+	-
Copano Bay, TX (CPB)	1	+	-
	3	+	-
Galveston Bay, TX (GB)	1	+	-
	2	+	-
	3	+	-
	4	+	+
	5	+	+
	6	+	+
Lake Madeline (Offatt's Bayou)	A	+	-
	B	I	?
Lavaca Bay, TX (LVB)	1	+	-
	2	+	-
	3	+	-
Nueces Bay, TX (NUB)	1	+	-
Port Lavaca, TX (PLV-PC)	1	+	-
Sabine Lake (SL or Sabine)	1A	I	?
	1B	I	?
	1C	+	+
	2A	+	+
	2B	I	?
	2C	I	?
	3A	I	?
	3B	I	?
	3C	+	-
Lake Madeline (Offatts Bayou)	4A	+	-
	4B	I	?
Nueces Bay (NUB)	1	+	-

[1] PCR product with universal bacterial primers 8f and 1492r.
[2] PCR product with *Dehalococcoides*-specific primers DET730f and 1492r.
[3] + denotes positive PCR product, - denotes negative PCR product.
[4] No PCR product indicating reaction inhibition.

Fig. 9. Map of Galveston Bay, TX, showing the Houston Ship Channel.

Fig. 10. Map of Sabine Lake, TX, showing the ship channel.

II. 3. 2. Extraction Method Justification

Dehalococcoides was detected in every core examined, except the wetlands control site, FW1A. Overall, 14 sediment cores were examined, of which three showed total PCR inhibition (only SG-1 included in the results). The remaining cores showed varying degrees of PCR inhibition. All in all, nucleic acids were extracted from 286 sediment samples. By looking at the results it seems that the extraction method used was not very efficient overall, since so many samples showed PCR inhibition. The extraction method

from Zhou et al (66) was developed for sediments with high organic and humic content. It was modified several times to increase its efficiency for the HSC samples. DNA precipitation times were changed; centrifugation times, speeds, and temperatures were varied; the chloroform isoamyl alcohol step was repeated to determine whether or not that would yield cleaner DNA. This extraction method was effective at extracting nucleic acids, unfortunately many PCR inhibitors were also co-extracted. This in turn is due to the sediment samples themselves. Since the HSC sediments are highly polluted, not just with dioxins, but with PCBs, hydrocarbons and oil residue, it is much more difficult to extract nucleic acids. After determining that this extraction method yielded 'dirty' DNA, commercial DNA extraction kits were tried. I chose three different kits from the manufacturer MoBio: Power Max Soil (MoBio Inc., Solana, CA), Power Soil (MoBio), and Ultra Clean Soil (MoBio). The Power Max Soil kit used 10g of frozen sediment, whereas the other two used one gram of sediment or less. Visually the DNA was cleaner; it was not brown in color as with the extraction method modified from Zhou et al (66). DNA concentrations were lower, but 260/280 absorbance ratios, indicating protein contamination, were better. Unfortunately, subsequent PCR reactions yielded no detectable product. The DNA concentrations for the Power Max Soil kit were extremely low, so the sample buffer volume was concentrated in a speed vacuum apparatus. However, subsequent PCR analysis yielded no product. After trying these various kits, it was decided that we would use the extraction method developed by Zhou et al (66) and try to clean the extracted nucleic acids as best we could. To my knowledge no one has extracted nucleic acids from sediment samples from the HSC, so there was

no published literature for advice. I also added a phenol-chloroform-isoamyl-alcohol step before the chloroform-isoamyl alcohol step in the extraction procedure and that also did not yield better results. Additional phenol extractions (with back-extractions) of samples that had already been extracted with the modified Zhou method, were attempted but did not reduce inhibition of PCR. In contrast, when I extracted the sediment samples collected from various Texas Bays, I used the same extraction method (modified from Zhou (66)) and I had almost no PCR inhibition. I was able to get PCR product from at least one sample from every bay system. Just from visual inspection, it was clear that the extracted nucleic acids were lighter in color, indicating that fewer inhibitors had been co-extracted. 260/280 absorbance ratios as well as 260/230 absorbance ratios, indicating organic and humic contamination of DNA, were better. I believe that no matter which extraction method is used, one will always have some degree of PCR inhibition with samples from the HSC, due to the high level of contaminants.

II. 3. 3. Primer Justification

As expected, *Dehalococcoides* was found in every core from the HSC and even in SG-8, the sediment core from across Galveston Bay. The primers used to determine the presence or absence of *Dehalococcoides* were DET730f (9) and the general bacterial primer 1492r (primer set 1). 1492r was chosen to increase the DNA fragment length. This primer set had been tested on library DNA from various bacterial groups (several proteobacteria and gram positive) available in the Brinkmeyer lab and did not produce a PCR product with any of them. However, as it turned out through sequencing analysis,

this primer set is not entirely *Dehalococcoides* specific. It is mostly specific for *Dehalococcoides,* but unfortunately it also amplifies a few other bacteria. However, the primer set DET730f and DET1350r (primer set 2) is entirely *Dehalococcoides* specific. The fact that primer set 1 is not entirely *Dehalococcoides* specific was not detected until sequencing analysis for a different part of this study was conducted. At this point, all of the samples had been extracted and tested for the presence or absence of *Dehalococcoides* using primer set 1. Subsequent analysis of other published *Dehalococcoides*-specific primer sets (20) in ARB, the sequencing software, revealed that if primer set 2 would have been used exclusively, not every *Dehalococcoides* sequence would have been detected that was present in the HSC sediment. So even though, primer set 1 showed some nonspecific amplification, primer set 2 would have missed some *Dehalococcoides* DNA fragments. I did try to amplify previously tested samples with primer set 2, however, these attempts were unsuccessful. This could be due to the fact that the DNA had been degraded and/or that PCR inhibitors were still present in the cleaned sample. Using primer set 2 to confirm that *Dehalococcoides* was present in SG-8 was successful because it was done within one week of the PCR analysis using primer set 1. The fact that *Dehalococcoides* was found in SG-8 with primer set 2 and the sequencing data from station 11270 in the HSC, which was amplified with primer set 1 (discussed in Chapter III), are strong indicators that the samples which showed presence of *Dehalococcoides* are not false positives.

III. 3. 4. Presence of *Dehalococcoides*

Yeager et al (64) showed that dioxin fluxes to sediments in the HSC remain orders of magnitude higher than atmospheric deposition (FW1A) and their sedimentary dioxin inventories are still orders of magnitude greater. Hence, it is not surprising that *Dehalococcoides* was detected in every sediment core, except the wetlands control site and the cores that had PCR inhibition throughout. The sediment cores collected were anoxic within the first centimeter and all of them had a distinct rotten egg/petroleum/ chemical smell to them-to varying degree. Since *Dehalococcoides* is an anaerobe, one would only expect it in anaerobic environments. Table 17 is a summary table showing the depths at which *Dehalococcoides* was first detected for each sediment core.

TABLE 17. Stations and the depth (in cm) at which *Dehalococcoides* was first detected.

Station	Sediment Depth (in cm) at which *Dehalococcoides* was first detected
11193	6
11270	3
15244	8
11261	30
13337	11
FW1A	Not detected
SG 1	Inhibition
SG 3	5
SG 4	2
SG 6	1
SG 7	3
SG 8	4

Detection of *Dehalococcoides* varied from 1cm (SG-6) to 30cm (11261). SG-6 1 cm could be a false positive, since primer set 1 did have some nonspecific amplification; however, sedimentary analysis revealed that the sediments of SG-6 had been mixed

recently, increasing the likelihood of the detection of *Dehalococcoides* in the upper sediment layers. *Dehalococcoides* was detected further down in the sediment core at depths 6cm through 10cm. Since the SG-6 sediment core was taken from the so called 'Dioxin Pit', it is very likely that *Dehalococcoides* is present already at 1 cm.

There seems to be a minimum dioxin concentration of about 3 total TEQ ng/kg dry weight needed for *Dehalococcoides* to occur. This is a low concentration compared to dioxin concentrations in the HSC sediments (station 11270: ~ 30 total TEQ ng/kg dry weight; stations 11193, 13337, SG 3, and SG 4 ~ 8 total TEQ ng/kg dry weight). *Dehalococcoides* was not detected in the wetlands control site (FW1A), where dioxin concentrations were below 1 total TEQ ng/kg dry weight. Table 18 shows the depths and (estimated) dioxin concentrations where *Dehalococcoides* was first detected.

TABLE 18. Stations, depths (in cm), and dioxin concentrations at which *Dehalococcoides* were first detected.

Station	**Sediment Depth (in cm) at which *Dehalococcoides* was first detected**	**Estimated Age of Sediment (yr)**	**Dioxin concentration (total TEQ ng/kg dry wt) at which *Dehalococcoides* was first detected**
11193	6	~ 2	~ 8
11270	3	3.48	~ 30
15244	8	7.12	~ 4-5
11261	30	5.2	2.98
13337	11	6.3	8.22
FW1A	Not detected	-	below 1
SG 1	Inhibition	-	-
SG 3	5	Data not yet available	~ 8-10
SG 4	2	Data not yet available	~ 7-8
SG 6	1	Data not yet available	Data not yet available
SG 7	3	Data not yet available	Data not yet available
SG 8	4	Data not yet available	Data not yet available

Dioxin concentrations vary widely and it seems that there is only a minimum of below 1 total TEQ ng/kg dry weight where *Dehalococcoides* does not occur. Since the detection of *Dehalococcoides* was based on molecular methods utilizing DNA-derived templates, one cannot validly conclude that the organisms were active and/or alive at all the depths at which they were detected, including the ones with very low dioxin concentrations. However, the fact that *Dehalococcoides* was extensively detected throughout all of the stations analyzed along the HSC makes it very likely that members of the group were indeed active in at least deeper parts of the sediment cores, i.e. the ones which exhibited higher dioxin concentrations. Bunge et al (14) showed that *Dehalococcoides* sp. strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-TCDD and 1,2,3,7,8-PeCDD. Fennell et al (15) showed that *Dehalococcoides ethenogenes* strain 195 is also able to dechlorinate 1,2,3,4-TCDD but is unable to dechlorinate 2,3,7,8-TCDD. Neither study investigated whether or not *Dehalococcoides* require a minimum or maximum concentration of substrate in order to thrive and grow. To my knowledge, no studies have been conducted to determine whether or not there is a concentration at which dioxins and other halogenated compounds become toxic to these bacteria. Since *Dehalococcoides* use dioxins and halogenated compounds as an energy source, this is unlikely (2).

There also seems to be no correlation with depth, since detection depths ranged from 2-30cm, rather the occurrence of *Dehalococcoides* seems to depend on as of yet unidentified factors. Sedimentary and biogeochemical data were not available for all

cores, making it difficult to link the presence of *Dehalococcoides* to metals, nutrients, etc. *Dehalococcoides* was detected at varying POC concentrations. *Dehalococcoides* was detected at very low POC concentrations indicating that *Dehalococcoides* is not dependent on POC as a carbon source for growth and replication. These results are consistent with other studies that have shown that *Dehalococcoides* use only hydrogen as an electron donor and chlorinated compounds as growth-supporting electron acceptors (2, 29, 33).

In every sample where *Dehalococcoides* were detected, hydrogen sulfide was also present. There also seems to be no direct correlation between chlorine, sulfate, iron, or manganese concentrations and the presence of *Dehalococcoides*. However, a trend does appear to exist between presence of *Dehalococcoides* and age of the sediment. For cores with data available for estimated age of sediments, *Dehalococcoides* is not detectable before 2 years (Table 18). I am still awaiting age data for the rest of the Sea Grant study cores to confirm this trend. If age of the sediments is the overriding factor for establishment of *Dehalococcoides* in the dioxin contaminated sediments, then this means that estimates of natural degradation rates must also take this 'establishment' period into account.

The fact that *Dehalococcoides* was sometimes not found at high dioxin concentrations may be explained by several factors. It is possible that *Dehalococcoides* was present below detection limits. Conventional PCR has a higher detection threshold compared to

real-time PCR. *Dehalococcoides* may be detectable in these samples via real-time PCR. Another reason for the absence of *Dehalococcoides* may be patchiness. Perhaps *Dehalococcoides* is not associated with each and every dioxin molecule, and so may not be covering every square centimeter of sediment. I did not find depth profiles of the presence or absence of *Dehalococcoides* in the published literature. So the fact that *Dehalococcoides* was sometimes not detected where expected may be due to specific factors that are not yet known.

III. 3. 5. Dredged versus Undredged

There was no PCR inhibition for all of the 18 samples used for this portion of my study. *Dehalococcoides* was found in both the dredged and undredged samples. It occurred in 8 out of 9 samples for the dredged sediment survey and only in 5 out of 9 for the undredged sediment survey. Assuming that there are no false positives these results are surprising since one would expect to find *Dehalococcoides* more in the undisturbed sediment. Overall, the dredged sediment samples had lower dioxin concentrations compared to the undredged samples. This could be due to the fact that dredging activity resuspends dioxins and other chemicals in the water column, thus reducing the dioxin concentrations in the sediment, but increasing them in the water column. Interestingly, *Dehalococcoides* seem to be able to withstand the dredging activity (i.e. mixing and introduction of oxygen) and remain in the sediment. However, since we detected DNA, it is possible that we detected the DNA from dead bacteria that are not active and growing anymore. So we cannot say whether or not dredging activity actually kills

Dehalococcoides or not. It is possible that some bacteria are killed during the dredging activity, but that parts of the microbial communities remain intact. Adrian et al (2000) showed that *Dehalococcoides* sp. strain CBDB1 is extremely oxygen sensitive, this is probably true for all *Dehalococcoides* strains, and if due to dredging activity *Dehalococcoides* were exposed to air, it would surely die. In conclusion, we detected *Dehalococcoides* in both the dredged and undredged sediment samples from the HSC.

III. 3.6. Texas Bays

Seven Texas Bay Systems, one additional port, and part of Offatt's Bayou, a tributary to Galveston Bay were examined for the presence of *Dehalococcoides* (See Texas Bays Table). Sediment grab samples were collected from each system and analyzed. *Dehalococcoides* was only found in two bay systems, Galveston Bay and Sabine Lake. Multiple grab samples were collected from these bay systems and *Dehalococcoides* was not detected in every sample. In Galveston Bay, *Dehalococcoides* was only detected at three stations, Trinity Bay and along the track of the HSC (Fig. 9). It was not detected at the three stations closest to the Gulf of Mexico. This leads to the conclusion that *Dehalococcoides* is migrating out of the HSC into upper Galveston Bay. Either it has not reached the lower bay yet or it is confined to fresher parts of Galveston Bay. Another reason could be that dioxin concentrations are too low in the lower part of Galveston Bay to support *Dehalococcoides* populations. A similar pattern was observed in Sabine Lake (Fig. 10). Again multiple samples were collected from this system and *Dehalococcoides* was only found at the most inland stations. Since Sabine Lake also has

a fairly busy harbor and petro-chemical complex, this pattern could be due to the same reasons as seen in Galveston Bay. *Dehalococcoides* was not found in Port Lavaca or other industrialized bays in Texas. This could be due to the fact that grab samples can only collect shallow sediments and we might have missed *Dehalococcoides* in those systems. Dioxin contamination in these bays has not been yet measured and maybe dioxin concentrations are too low to support *Dehalococcoides* populations.

III. 3. 7. Conclusions

It appears that *Dehalococcoides* needs four things to occur in the sediment:

1.) greater than 3 TEQ ng/kg dry weight dioxin concentrations.

2.) presence of detectable hydrogen sulfide.

3.) sediment which has accumulated for 2 years or more

4.) presence of POC, at greater or equal to 0.4%.

(this actually indicates that *Dehalococcoides* is independent of POC concentration)

The fact that *Dehalococcoides* is present in the HSC and the upper part of Galveston Bay and Sabine Lake is very promising in terms of bioremediation. Incubation studies with environmental samples have shown that *Dehalococcoides* can dechlorinate dioxins and other chemicals, such as PCBs (1, 2, 9, 14, 22). Incubation experiments with dioxin contaminated HSC sediments are currently underway at TAMUG using various carbon sources to accelerate dechlorination rates and should provide insights for accelerating *in situ* bioremediation throughout the HSC.

Bioremediation in the HSC is favorable compared to dredging and depositing the sediment in landfills as well as chemical remediation, which would hinder the ship traffic. It will be interesting to see how this bioremediation effort will be conducted, since jurisdiction of channel waters is unclear. The Port of Houston Authority claims responsibility for the sediments, but not the water above. Hence it could argue that since it is not responsible for the water, it is not responsible for dioxin reaching the sediment. Most of the dioxin offenders in the HSC are historic, such as paper mills which have long since closed down and no longer exist. Do state or even federal agencies pick up these sites? Can one turn the HSC into a superfund site with billions of dollars on the line? Regardless of who will end up doing it, bioremediation is definitely the best way to go for the HSC in terms of dioxin contamination.

These results support the use of *Dehalococcoides* as a biological proxy for dioxin contamination. *Dehalococcoides* was detected at dioxin concentrations ranging from 3 to 239 total TEQ ng/kg dry weight (in HSC sediments), but not at concentrations below 1 total TEQ ng/kg dry weight (wetlands control site). Hence when *Dehalococcoides* was detected in the sediment using molecular methods, the dioxin concentrations were above the background levels of atmospheric deposition. Screening for the presence of *Dehalococcoides* in sediments is a fast and inexpensive way to determine contamination with dioxins. Typical dioxin analysis of sediments in the U.S. costs approximately $1,500, whereas a PCR reaction only costs about $6. Besides being less expensive,

screening for *Dehalococcoides* is also faster. Dioxin analysis can take up to several months, whereas PCR analysis can be completed within a few weeks or even days.

CHAPTER III

BACTERIAL DIVERSITY IN SEDIMENTS FROM THE HOUSTON SHIP CHANNEL

III. 1. Introduction

III. 1. 2. Houston Ship Channel

The Houston Ship Channel (HSC), located in the San Jacinto River Basin, in the northwest corner of Galveston Bay, Texas, is 50 miles in length, extending from the Port of Houston to the Gulf of Mexico (Ch. I, Fig. 1). The Port of Houston is the sixth largest seaport in the world and handles more foreign water-borne tonnage than any other U.S. port. The Port of Houston generates over $10 billion annually and each year more than 6,300 vessels pass through the HSC. The HSC is also home to the largest petrochemical complex in the United States and the second largest worldwide (43). The HSC is continually being dredged and the dredged sediment is used to create spoil islands and wetlands. Both the HSC and upper Galveston Bay (GB) are highly polluted with dioxins, dioxin-like compounds, and many other contaminants, such as hydrocarbons, from industrial and municipal effluents and runoff, as well as from atmospheric wet and dry deposition. In 1990, dioxins were detected in fish and crab tissue obtained from the HSC. A seafood consumption advisory for catfish and blue crabs was issued for the HSC and upper GB, and remains in effect to this day. Subsequently, the HSC was placed on the §303 (d) list of impaired water bodies as required by the 1977 Clean Water Act (as amended, 1996) and a total maximum daily loads (TMDL) study was initiated by the

Texas Commission on Environmental Quality (TCEQ). The study revealed that Toxic Equivalent (TEQ) concentrations in water ranged from 0.10 to 3.16 pg TEQ/L and in bottom sediments from 0.9 to 139.8 ng/kg dry wt. (57). On average, dioxin concentrations exceeded the Texas Surface Water Quality Standard (0.093 pg/L) in more than 80% of all samples (48). The study also revealed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the major contributor to total TEQs in all samples. The entire HSC is contaminated with dioxins and recent dioxin inputs to the HSC continue despite regulatory efforts (64).

III. 1. 3. Dioxins

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (dioxins) are persistent environmental contaminants. Dioxins cause a variety of biochemical, immunological, and reproductive effects in animals and are suspected carcinogens (6, 7, 28, 42, 46, 51). Dioxins bioaccumulate in the aquatic and terrestrial food chains posing significant and persistent risks to human health. The estimated half-life of dioxin in the human body is 7-8 years (58). Primary sources of dioxins include the production of herbicides (56), paper and pulp bleaching, metal smelting, and waste incineration (16, 18, 54, 62). Dioxins and dioxin-like compounds are hydrophobic and therefore have a high particle and lipid affinity. Their water solubility is estimated to be 19.3 ng/L (58). Due to their high hydrophobicity, dioxins present in the water column rapidly partition to organic carbon fractions (i.e black carbon) in suspended soils and can subsequently be buried in sediments (11, 38, 52). Re-suspension of polluted sediments

may re-introduce dioxins into the aquatic food chain; however, this process has not been thoroughly investigated. From both fiscal and environmental perspectives, in situ microbial remediation of dioxins in the HSC and GB is preferable to alternatives, such as removal of contaminated sediments to landfills or chemical treatments. Microbial remediation would also not interfere with the vessel traffic through the HSC. Since the HSC is tidally influenced, dioxin contamination has been transferred up- and downstream of the channel, increasing the urgency of remediation.

III. 1. 4. Microbial Dechlorination

Studies of microbial dechlorination of polychlorinated compounds have been mostly limited to freshwater systems and have indicated that degradation rates are enhanced under anaerobic, reducing conditions (1). Quensen et al. (44) showed that the chlorinated compound DDE (1,1-dichloro-2,2,-bis(p-chlorophenyl)ethylene), a commercial by-product in DDT formulations, is preferentially degraded under methanogenic and sulfidogenic conditions. Another study found that 2,3,7,8-TCDD was degraded up to 86% under anaerobic, reducing conditions (25) (Ch. I; Fig. 2). The reductive dehalogenation of chlorinated aromatic compounds has been identified as an energy-yielding process in a number of anaerobic bacteria (22). These anaerobic bacteria use polychlorinated compounds as electron acceptors and hydrogen as an electron donor (2, 14, 22). Identification of reductively dechlorinating bacteria has typically been limited to amended microcosm experiments. These dechlorinators include the low GC Gram-positive bacteria (*Desulfitobacterium* and *Dehalobacter*), members of the *Proteobacteria*

such as *Desulfomonile*, *Desulfuromonas* and *Dehalospirillum*, and finally the *Chloroflexi* also known as the green non-sulfur bacteria (4, 33, 22, 63). Another bacterial group, *Dehalococcoides*, is also known to reductively dechlorinate highly chlorinated compounds, making the resulting congeners and other biproducts more susceptible to degradation by other bacterial groups. The closest phylogenetic affiliation of *Dehalococcoides* is with the green non-sulfur bacteria or *Chloroflexi* (20, 24, 60), however there is increasing evidence that they may constitute a new division of bacteria (22, 33). To date, all *Dehalococcoides* isolates that have been studied are obligate dehalorespirers, using halogenated aliphatic or aromatic compounds as electron acceptors (63). Thus far, *Dehalococcoides* have mainly been isolated from groundwater and other freshwater systems (14), although more recent studies have isolated *Dehalococcoides*-like species from estuarine sediments (4). *Dehalococcoides ethenogenes* strain 195, isolated from contaminated groundwater, is the only known isolated organism capable of fully dechlorinating tetrachloroethene (PCE) and other chloroethenes to the non-toxic end-product ethene (2). Strains FL2 (isolated from a highly enriched PCE-to-ethene dechlorinating mixed culture from Red Cedar River sediment, Michigan, Loeffler et al, 2000) and DCEH2 (isolated from a dechlorinating enrichment mixed culture, GenBank accession number AJ249262) also dechlorinate chloroethenes (20). Strain CBDB1 (isolated from an enriched chlorobenzene-dechlorinating mixed culture from Saale River sediment, Germany) dechlorinates trichlorobenzenes and tetrachlorobenzenes to dichlorobenzenes, but is unable to dechlorinate PCE or trichloroethene (2). Strain CBDB1 is also able to dechlorinate

chlorinated benzenes (14). Members of *Dehalococcoides* have also been shown to dechlorinate commercial polychlorinated biphenyls (PCBs) (i.e Aroclor 1260) (9). Bedard (9) also found that *Dehalococcoides* obtain energy for growth from dechlorination. In 2003, Bunge et al (14) showed that strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD). In 2004, Fennell et al (15) showed that *Dehalococcoides ethenogenes* strain 195 is also able to dechlorinate 1,2,3,4-TCDD. However, *D. ethenogenes* strain 195 is unable to dechlorinate 2,3,7,8-TCDD, even though it has been found that its genome contains up to 17 putative dehalogenase gene homologues (53). Since the dehalogenation of dioxin by *Dehalococcoides* is orders of magnitude faster (weeks versus 1 to 4 years) than its anaerobic co-metabolic reduction (1, 14) its presence in or addition to dioxin contaminated areas is a significant contribution for bioremediation (1, 2, 14, 22).

III. 1. 5. Objective

I constructed 16S rRNA gene clone libraries to determine total bacterial diversity as well as the diversity of dioxin-degrading bacteria, especially *Dehalococcoides.* Different sampling locations within the Houston Ship Channel and a control site as well as different sediment depths were characterized to determine the impact of dioxin-respiring bacteria on total bacterial diversity.

Hypothesis 2: I expect the bacterial diversity in the HSC sediments to be skewed towards toxin-degrading bacteria, such as *Dehalococcoides.*

I tested this hypothesis by constructing 16S rRNA gene clone libraries to determine bacterial diversity. Community DNA was extracted from sediment samples and PCR was performed on the extracted DNA. Carefully chosen samples were cloned and analyzed with restriction enzyme digests. Clones were sequenced to determine bacterial diversity as well as diversity within dioxin-degrading bacteria.

III. 2. Materials and Methods

III. 2. 1. Sampling

Sediment cores were collected along the HSC and at a freshwater control site (Ch. I; Fig. 3 and Table 1). Only sediment cores which showed minimal signs of mixing, as determined by X-radiographs, were selected for analysis. Sediment cores were collected as previously described by Yeager et al (64). Each core was sectioned at 1cm intervals over the upper ~50cm and at 2cm intervals thereafter. Sterile technique was applied to every extent possible. Aliquots were collected with an ethanol-flamed spatula, transferred into 50ml sterile Falcon tubes, and frozen at -20°C until later analysis.

For this part of my thesis, sediment samples were selected for the determination of bacterial diversity as well as diversity within dioxin-degrading bacteria based upon dioxin concentration and depth of the core i.e. age of the sediment layer. In order to

determine the total bacterial diversity in HSC sediments, we selected Station 11270 because it had the highest dioxin concentrations out of all of the HSC cores analyzed for dioxin at that time. Within this sediment core, we selected two depths for bacterial diversity analysis. We selected 3cm since it was the first depth at which *Dehalococcoides* was detected with PCR analysis. The estimated age of this layer was 3.48 yr and concentrations of 2,3,7,8-TCDD and 2,3,7,8-TCDF were approximately 26.00 and 57.00 ng/kg dry wt, respectively. We also chose 20cm because of its estimated age, 23.2 yr, and higher concentrations of 2,3,7,8-TCDD (96.00 ng/kg dry wt) and 2,3,7,8-TCDF (230.00 ng/kg dry wt). *Dehalococcoides* had also been detected at this depth with PCR analysis. Both of these depths were anaerobic. We also wanted to compare the diversity of HSC sediments to sediments from the wetlands control site (FW1A). Due to PCR inhibition we could not compare 3cm, but were able to compare 20cm. For all the samples used in this part of the thesis see Table 19.

TABLE 19. Sample stations and depths analyzed for this part of the thesis.

Station	Depth (cm)	Total Diversity	*Dehalococcoides* Diversity
11270 (HSC)	3cm	X	X
11270 (HSC)	20cm	X	X
FW1A	20cm	X	--

III. 2. 2. Extraction of Nucleic Acids

Nucleic acids from environmental sediment samples were extracted using the protocol previously described by Zhou et al (66). The Zhou et al (66) protocol was slightly

modified for our experiments. 5g of frozen sediment was weighted out in a 50ml sterile Falcon tube. 13.5ml extraction buffer and 100µl (10mg/ml) of the enzyme Proteinase K were added to the sediment. The enzyme Proteinase K is often used to lyse bacterial cells. The Falcon tubes were placed in a shaking (240 rpm) 37°C water bath and incubated for 30 minutes. After the shaking water bath, 1.5ml 20% sodium dodecyl sulfate (SDS) was added and the tubes were put in a 65°C water bath for 2 hours. After the 2 hour incubation period, the sediment was centrifuged at 6,500 x g, for 10 minutes at room temperature (20°C). The supernatant was transferred into a new Falcon tube with a sterile Pasteur pipet. The sediment pellet was extracted two more times by adding 4.5ml extraction buffer and 0.5ml 20% SDS. The tubes were briefly vortexed to loosen the pellet and incubated at 65°C for 10 minutes. The tubes were centrifuged as before and the supernatant was combined. Following the pellet extraction was a chloroform cleaning step. An equal volume of chloroform isoamyl alcohol (24:1) was added to the supernatant. The tubes were inverted until an emulsion formed and centrifuged at 6,500 x g for 15 minutes at room temperature (20°C). The upper aqueous phase was removed with a Pasteur pipet and transferred into a new sterile 50ml Falcon tube. This was followed by isopropanol precipitation. 0.6 volume of cold (-20°C) 100% isopropanol was added to the aqueous phase. The tubes were inverted and the DNA was allowed to precipitate in the freezer overnight. The next day the tubes were centrifuged at 6,500 x g for 1 hour at 4°C. The supernatant was decanted and the DNA pellet washed with 80% ice-cold ethanol to remove any remaining salts. About 1ml of ice-cold 80% ethanol was added to the pellet and the tubes were centrifuged again for 5 minutes (6,500 x g and

4°C). The DNA pellets were allowed to air dry overnight. The next day they were resuspended in 500µl LT buffer. Once the samples were resuspended, they were transferred to 2ml Eppendorf tubes and centrifuged at 6,500 x g for 15 minutes at 4°C. The supernatant was transferred to a new 2ml tube and stored at -20°C for later PCR analysis.

Extracted nucleic acids had to be cleaned with the WIZARD DNA Cleanup System (Promega Corp., Madison, Wis.) according to the manufacturer's protocol. Most times the eluate from the first minicolumn was purified further with fresh resin and passage through another minicolumn. This second purification step was necessary to remove PCR inhibitors that were coextracted from the samples. The DNA concentrations and the 260/280 absorbance ratios were recorded for each DNA sample after extraction.

III. 2. 3. PCR Amplification of 16S rRNA Gene

16S rRNA gene sequences were amplified from the environmental sediment sample nucleic acid extracts by PCR with an automated thermal cycler (Eppendorf, Hamburg, Germany) by using the bacterium-specific primers 8*f* and 1492*r*. The PCR reactions were 50µl in volume and contained 0.5µl of each primer (10µmol), 1µl dNTPs (10mmol), 1µl BSA, 5µl PCR buffer (Roche, Basel, Switzerland), 0.5µl Taq (Roche). Final volume was reached with PCR water. From 1µl to 5µl of DNA (100 ng/ µl) was added to the final reaction mixture. PCR conditions were as follows: initial denaturation step at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 50°C for 1

minute, and 72°C for 3 minutes, followed by a final extension at 72°C for 10 minutes. 10µl of the PCR products were run on a 1% agarose gel at 100V, stained with ethidium bromide, and visualized under UV light. PCR products were purified with the WIZARD PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol. Using a more specific primer set (DET730f and 1492r) under the conditions previously described by Breitenstein et al (10), a nested PCR was performed on the initial PCR product to confirm the presence or absence of the bacterial group *Dehalococcoides*. PCR reactions were the same as described above, but with different primers. Only cleaned PCR products were used for cloning.

III. 2. 4. 16S rRNA Gene Clone Library Construction

16S rRNA gene clone libraries were constructed with the pGEM-T-Easy vector system (Promega). The pGEM-T-Easy vector system is a convenient system for the cloning of PCR products. The vector for this particular system has a 3´ terminal thymidine at both ends. These 3´-T overhangs at the insertion site (of the DNA fragment) greatly improve ligation efficiency by preventing recircularization of the vector (45). The Taq DNA polymerase (Roche) used in our PCR reactions added a single 3´ terminal adenosine to the ends of the amplified fragments. The fact that both the vector and the DNA fragment have so called "sticky ends" enhances ligation efficiency as well. The pGEM-T-Easy vector also contains a α-peptide coding region. The α-peptide is inactivated when a DNA fragment is inserted into the vector and this inactivation allows recombinant clones to be identified by color screening on indicator plates (45).

The ligation reactions were set up as follows: 5µl 2x Rapid Ligation Buffer T4 DNA Ligase, 1µl pGEM-T-Easy Vector (50ng), 2µl cleaned PCR product (bright band on agarose gel) or 3µl cleaned PCR product (weak band on agarose gel), 1µl T4 DNA Ligase (3 Weiss units/µl), and PCR water to a final volume of 10µl. The reactions were mixed by pipetting and incubated overnight at 4°C for maximum number of transformants.

JM109 High Efficiency Competent Cells (Promega) were used for the transformations. It is essential to use competent cells with a transformation efficiency of at least 10^8 cfu/µg (cfu = colony forming units) in order to obtain a reasonable number of colonies. JM109 cells are guaranteed to have a transformation efficiency of at least 10^8 cfu/µg. Ligation reaction tubes were centrifuged to collect contents at the bottom. 2µl of each ligation reaction was added to a 15ml sterile Falcon tube on ice. JM109 High Efficiency Competent Cells (Promega) were removed from the -80°C freezer and thawed for approximately five minutes. 50µl of cells were carefully transferred into each 15ml sterile Falcon tube. 15ml tubes were gently petted to mix and placed on ice for 20 minutes. The cells were then heat-shocked for 50 seconds in a water bath at exactly 42°C. The tubes were immediately returned to ice for two minutes. 950µl room temperature SOC medium (100ml contains 2g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} stock (filter-sterilized), and 1ml 2M glucose (filter-sterilized)) was added to each tube. Tubes were incubated for 1.5 hours at 37°C with shaking (150rpm). 100µl of each transformation culture were plated onto

duplicate or triplicate LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature). These particular agar plates allowed for color screening of the recombinant clones. The plates were incubated overnight at 37°C. The next day, the plates were color screened. White colonies contained the vector insert and blue colonies did not. The largest white colonies were transferred with sterile toothpicks onto fresh LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature) and incubated overnight at 37°C. The next day, half of each colony was transferred with a sterile toothpick into 0.5ml tubes containing 24µl "2 x cracking buffer" (50 ml contain 1ml 5M NaOH, 1ml 0.5M EDTA, 5ml 10% SDS, 5ml 100% Glycerol, and 38ml deionized water). The "cracking buffer" digested the competent cells, hence releasing the plasmid (vector containing DNA fragment). The entire 24µl were run on a 1% agarose gel at 100V, stained with ethidium bromide, and visualized under UV light. This step allowed us to check that the DNA fragment inserted into the vector was of correct size. The remaining halves of each colony were incubated again overnight at 37°C and transferred with a sterile toothpick onto fresh LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature). The selected colonies were transferred with a sterile flaming loop to 15ml sterile Falcon tubes containing 5ml LB broth. The tubes were incubated overnight at 37°C with shaking (150rpm) until cloudy. The next day, the tubes were centrifuged at 4000 x g for two minutes at room temperature to collect the cell pellet. The supernatant was decanted and 1ml of 50mM EDTA (ethylenediaminetetraacetic acid) was added to wash the cell pellet. The cell pellet was resuspended by pipetting and centrifuged as before. The supernatant was decanted and the cell pellet was stored at -20°C until

plasmid extraction. The plasmids were extracted using the E.Z.N.A. Plasmid Extraction Kit (Omega BioTek, Atlanta, GA). The plasmids were extracted according to the manufacturer's protocol. The extracted plasmids containing the desired DNA fragment were stored at -20°C for later analysis. PCR was performed to ensure that the extracted plasmids contained the 16S rDNA fragment and that they were pure enough for downstream analyses. The PCR reactions were 50µl in volume and contained 45µl PCR Supermix (Invitrogen, Carlsbad, CA), 0.5µl of primer 8*f* (10µmol), 0.5µl of primer 1492*r* (10µmol),1µl BSA, 2µl PCR water, and 1µl plasmid DNA. PCR conditions were the same as previously described.

III. 2. 5. ARDRA

Each individual clone was subjected to amplified ribosomal DNA restriction analysis (ARDRA) in order to characterize the 16S rRNA gene diversity within each clone library (32, 59). ARDRA reactions were as follows: 10µl PCR product (plasmid DNA), 0.75µl HaeIII restriction enzyme (7.5 units) (Promega), 0.75µl RsaI restriction enzyme (7.5 units) (Promega), 1.5µl 10x Buffer C (Promega), 0.15µl BSA (Promega), and 1.85µl PCR water for a final reaction volume of 15µl. ARDRA reactions were vortexed and centrifuged briefly and incubated 37°C for 4 hours. The resulting ARDRA patterns were separated on an 8% acrylamide gel [19:1, acrylamide / bis-acrylamide] using the BIORAD D-Code DGGE system (BioRAD, Hercules, CA). The pGEM DNA Marker (Promega) was used as the standard size ladder. The gels were run at 120V and 40°C for approximately 3 hours. The gels were stained with ethidium bromide and visualized

under UV light. ARDRA patterns were analyzed using the GelCompar software program (Applied Maths, Inc., Austin, TX). The cluster analysis method used was the comparative numerical analysis with the unweighted pair group method using arithmetic averages (UPGMA). Based on this cluster analysis one or in some cases several representatives of each ARDRA pattern group from all clone libraries were selected for sequencing.

III. 2. 6. Sequencing and Phylogenetic Analysis

Sequencing was performed at the DNA Analysis Facility on Science Hill at Yale University. Sequence data were first "blasted" in GenBank to identify the most similar sequences and then analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). This software is commonly used when analyzing 16S rRNA gene diversity. Dendrograms were reconstructed for the phylogenetic analysis. The frequencies of 16S rRNA gene phylotypes determined by ARDRA and subsequent sequencing (i.e., those sharing >97% identity) were used for analysis of diversity. Shannon's index for diversity (H') was calculated according to the method of Zar (65). Shannon's index for diversity is by far the most commonly used diversity index. It takes into account the number of species but also the abundance of each species. Rarefaction curves were interpolated with the freeware program Analytic Rarefaction 1.3 (http://www.uga.edu/_strata/software/index.html). Rarefaction allows one to calculate species richness for a given number of sampled individuals. Rarefaction curves show the number of species as a function of the number of individuals sampled.

Hence, a steep slope indicates that a fraction of the species diversity has not been sampled whereas a flattening slope indicates that diversity has been sampled well. Coverage of the clone libraries was estimated as described by Mullins et al. (39). Coverage was derived from the equation

$$C = 1 - (n_1/N)$$

where, n_1 is the number of clones that occurred only once and N is the total number of clones examined. This value is conservative, but excludes variation introduced by PCR artifacts and heterogeneities in rDNA gene families (39).

III. 3. Results and Discussion

III. 3. 1. Diversity within *Dehalococcoides* from HSC Sediments

The diversity of *Dehalococcoides* was characterized at one sampling location within the HSC and at 3 and 20 cm. We selected Station 11270 because it had the highest dioxin concentrations out of all of the HSC sediment cores analyzed for dioxin at that time. Within this sediment core, we selected two depths for *Dehalococcoides* diversity analysis based upon estimated age and dioxin concentration.

Tables 20, 21, and 22 show the results for the diversity of *Dehalococcoides* at 3cm, 20cm, and both respectively. Figs. 11, 12, and 13 show the phylogenetic trees for the diversity of *Dehalococcoides* at 3cm, 20cm, and both respectively.

TABLE 20. Diversity of *Dehalococcoides* 16S rRNA clones at Station 11270 at 3cm depth.

Closest 16S rRNA relative identified in GenBank and ARB database	**% Similarity**	**Number of Clones**
Dehalococcoides ethenogenes 195 / spp. strain CBDB1	99	11
Uncultured bacterium clone FS117-51B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	16
Uncultured bacterium clone FS142-4B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	2
Uncultured bacterium clone FS117-42B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	90	1
Uncultured *Chloroflexi* bacterium clone MSB-5bx1 in mangrove soil	95	1
Uncultured *Chloroflexi* bacterium clone MSB-4E3 in mangrove soil	92	3
Uncultured bacterium clone S2 from a chlorinated ethene contaminated aquifer (*Chloroflexi*)	96	2
Uncultured bacterium clone: QpjB72fl; *Chloroflexi*-like bacterium in sludge	94	1
Uncultured bacterium clone AKIW460 (*Firmicutes, Bacillales; Planococcaceae, Sporosarcina*) (similar to bacteria found in aerosols in Austin and San Antonio)	99	1
Geosporobacter subterrenus strain VNs68, from a deep subsurface aquifer (*Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Geosporobacter*)	92	2
Uncultured bacterium clone Nubeena383 from organically-enriched fish farm sediments (*Gammaproteobacteria*)	90	1
Desulfobacterium anilini strain AK1 (*Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; Desulfobacterium anilini*)	95	1
Uncultured delta proteobacterium clone: GuBH2-AD/TzT-67 found in uranium waste soil	97	2
Uncultured bacterium clone CR99-2-75 from the Changjiang River, Japan (*Deltaproteobacteria*)	97	1
Uncultured hydrocarbon seep bacterium GCA025 (similar to *Candidate Division JS1*)	99	2
Uncultured bacterium clone VHS-B5-64 from Victoria Harbor sediments, Hong Kong (*Candidate Division OP11*)	93	1
Uncultured candidate division OP11 bacterium clone MSB-4D8 in mangrove soil	89	1
Uncultured bacterium clone 35-52 bacterium from sediment from Guanting Reservoir, China (*Nitrospirae*)	95	2
Uncultured *Deferribacteres* bacterium clone MSB-4A2 in mangrove soil	94	1
Uncultured *Bacteroidetes* bacterium clone MSB-5A10 in mangrove soil	99	1
Uncultured forest soil bacterium clone DUNssu128 (*Planctomycete*)	89	2
Uncultured spirochete clone MS12-6-B11 from a hydrothermal vent in New Zealand	89	2
Uncultured bacterium clone: Y160 in coastal sediment near shellfish aquaculture (unknown)	93	1
Uncultured bacterium clone: KY177 in coastal sediment near shellfish aquaculture (unknown)	96	1

TABLE 21. Diversity of *Dehalococcoides* 16S rRNA clones at Station 11270 at 20 cm depth.

Closest 16S rRNA relative identified in GenBank and ARB database	**% Similarity**	**Number of Clones**
Dehalococcoides ethenogenes 195/ spp. strain CBDB1	99	10
Dehalococcoides sp. BAV1	91	3
Uncultured *Dehalococcoides sp.* clone ccslm202 at a TCE-contaminated site	94	2
Uncultured bacterium clone FS117-51B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	1
Uncultured bacterium clone FS142-4B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	2
Uncultured *Chloroflexi* bacterium clone MSB-5bx1 in mangrove soil	95	13
Uncultured *Chloroflexi* bacterium clone MSB-5A8 in mangrove soil	92	1
Uncultured *Chloroflexi* bacterium clone MSB-4E3 in mangrove soil	96	1
Uncultured *Chloroflexi* bacterium clone TK-SH11 from Lake Tanganyika in Central Africa	86	1
Uncultured bacterium clone S2 from a chlorinated ethene contaminated aquifer (*Chloroflexi*)	96	2
Uncultured bacterium clone: QpjB72fl; *Chloroflexi*-like bacterium in sludge	99	6
Uncultured bacterium clone MB-C2-127 in methane-hydrate bearing deep marine sediments (*Chloroflexi*)	94	2
Uncultured bacterium clone AKIW460 similar to bacteria found in aerosols in Austin and San Antonio (*Firmicutes, Bacillales; Planococcaceae, Sporosarcina*)	99	9
Uncultured bacterium clone ATB-KM1285 from biogas-producing reactor (*Firmicutes, Bacillales; Planococcaceae, Sporosarcina*;)	92	4
Sporosarcina sp. 3061 from Pacific deep sea sediment (*Firmicutes; Bacillales; Planococcaceae, Sporosarcina*)	92	1
Sporosarcina sp. S11-2 from Arctic Ocean sediments (*Firmicutes; Bacillales; Planococcaceae; Sporosarcina*)	98	1
Uncultured soil bacterium clone M09_Pitesti from oil polluted soil in Romania (*Firmicutes, Clostridia*)	97	1
Uncultured hydrocarbon seep bacterium GCA025 (similar to *Candidate Division JS1*)	94	1
Uncultured actinobacterium clone: Y57 in coastal sediment near shellfish aquaculture	84	1
Uncultured bacterium clone: Y160 in coastal sediment near shellfish aquaculture (unknown)	92	1

TABLE 22. Diversity of *Dehalococcoides* 16S rRNA clones at station 11270 at 3cm and 20cm depths.

Closest 16S rRNA relative identified in GenBank and ARB database	**% Similarity**	**Number of Clones**
Dehalococcoides ethenogenes 195/ spp. strain CBDB1	99	21
Dehalococcoides sp. BAV1	91	3
Uncultured *Dehalococcoides sp*. clone ccslm202 at a TCE-contaminated site	94	2
Uncultured bacterium clone FS117-51B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	17
Uncultured bacterium clone FS142-4B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	91	4
Uncultured bacterium clone FS117-42B-02 (*Dehalococcoides*-like bacterium in ridge flank crustal fluids)	90	1
Uncultured *Chloroflexi* bacterium clone MSB-5bx1 in mangrove soil	95	14
Uncultured *Chloroflexi* bacterium clone MSB-5A8 in mangrove soil	92	1
Uncultured *Chloroflexi* bacterium clone MSB-4E3 in mangrove soil	94	4
Uncultured *Chloroflexi* bacterium clone TK-SH11 from Lake Tanganyika in Central Africa	86	1
Uncultured bacterium clone S2 from a chlorinated ethene contaminated aquifer (*Chloroflexi*)	96	4
Uncultured bacterium clone: QpjB72fl; *Chloroflexi*-like bacterium in sludge	97	7
Uncultured bacterium clone MB-C2-127 in methane-hydrate bearing deep marine sediments (*Chloroflexi*)	94	2
Uncultured bacterium clone AKIW460 similar to bacteria found in aerosols in Austin and San Antonio (*Firmicutes, Bacillales; Planococcaceae, Sporosarcina*)	99	10
Uncultured bacterium clone ATB-KM1285 from biogas-producing reactor (*Firmicutes, Bacillales; Planococcaceae, Sporosarcina*)	92	4
Sporosarcina sp. 3061 from Pacific deep sea sediment (*Firmicutes; Bacillales; Planococcaceae, Sporosarcina*)	92	1
Sporosarcina sp. S11-2 from Arctic Ocean sediments (*Firmicutes; Bacillales; Planococcaceae; Sporosarcina*)	98	1
Uncultured soil bacterium clone M09_Pitesti from oil polluted soil in Romania (*Firmicutes, Clostridia*)	97	1
Geosporobacter subterrenus strain VNs68, from a deep subsurface aquifer (*Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Geosporobacter*)	92	2
Uncultured bacterium clone Nubeena383 from organically-enriched fish farm sediments (*Gammaproteobacteria*)	90	1
Desulfobacterium anilini strain AK1 (*Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae; Desulfobacterium anilini*)	95	1
Uncultured delta proteobacterium clone: GuBH2-AD/TzT-67 found in uranium waste soil	97	2
Uncultured bacterium clone CR99-2-75 from the Changjiang River, Japan (*Deltaproteobacteria*)	97	1
Uncultured hydrocarbon seep bacterium GCA025 (similar to *Candidate Division JS1*)	94	1
Uncultured hydrocarbon seep bacterium GCA025 (similar to *Candidate Division JS1*)	99	2

TABLE 22. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	**% Similarity**	**Number of Clones**
Uncultured bacterium clone VHS-B5-64 from Victoria Harbor sediments, Hong Kong (*Candidate Division OP11*)	93	1
Uncultured candidate division OP11 bacterium clone MSB-4D8 in mangrove soil	89	1
Uncultured actinobacterium clone: Y57 in coastal sediment near shellfish aquaculture	84	1
Uncultured bacterium clone 35-52 bacterium from sediment from Guanting Reservoir, China (*Nitrospirae*)	95	2
Uncultured *Deferribacteres* bacterium clone MSB-4A2 in mangrove soil	94	1
Uncultured *Bacteroidetes* bacterium clone MSB-5A10 in mangrove soil	99	1
Uncultured forest soil bacterium clone DUNssu128 (*Planctomycete*)	89	2
Uncultured spirochete clone MS12-6-B11 from a hydrothermal vent in New Zealand	89	2
Uncultured bacterium clone: Y160 in coastal sediment near shellfish aquaculture (unknown)	92	1
Uncultured bacterium clone: Y160 in coastal sediment near shellfish aquaculture (unknown)	93	1
Uncultured bacterium clone: KY177 in coastal sediment near shellfish aquaculture (unknown)	96	1

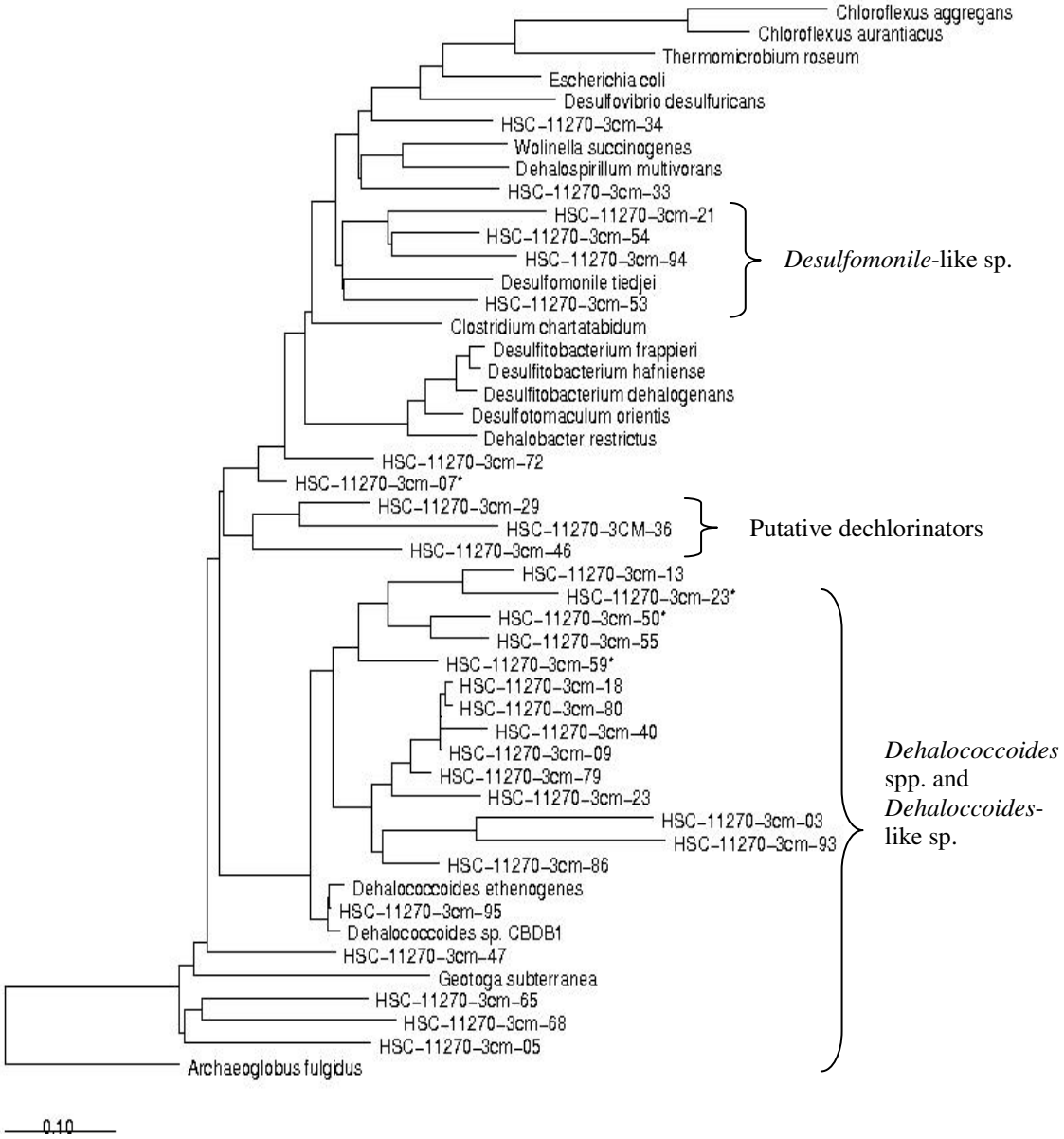

FIG. 11. Diversity of *Dehalococcoides* at 3cm (Station 11270). * indicates that bacterium was found at both depths (3cm and 20cm). The scale bar represents 10% sequence divergence.

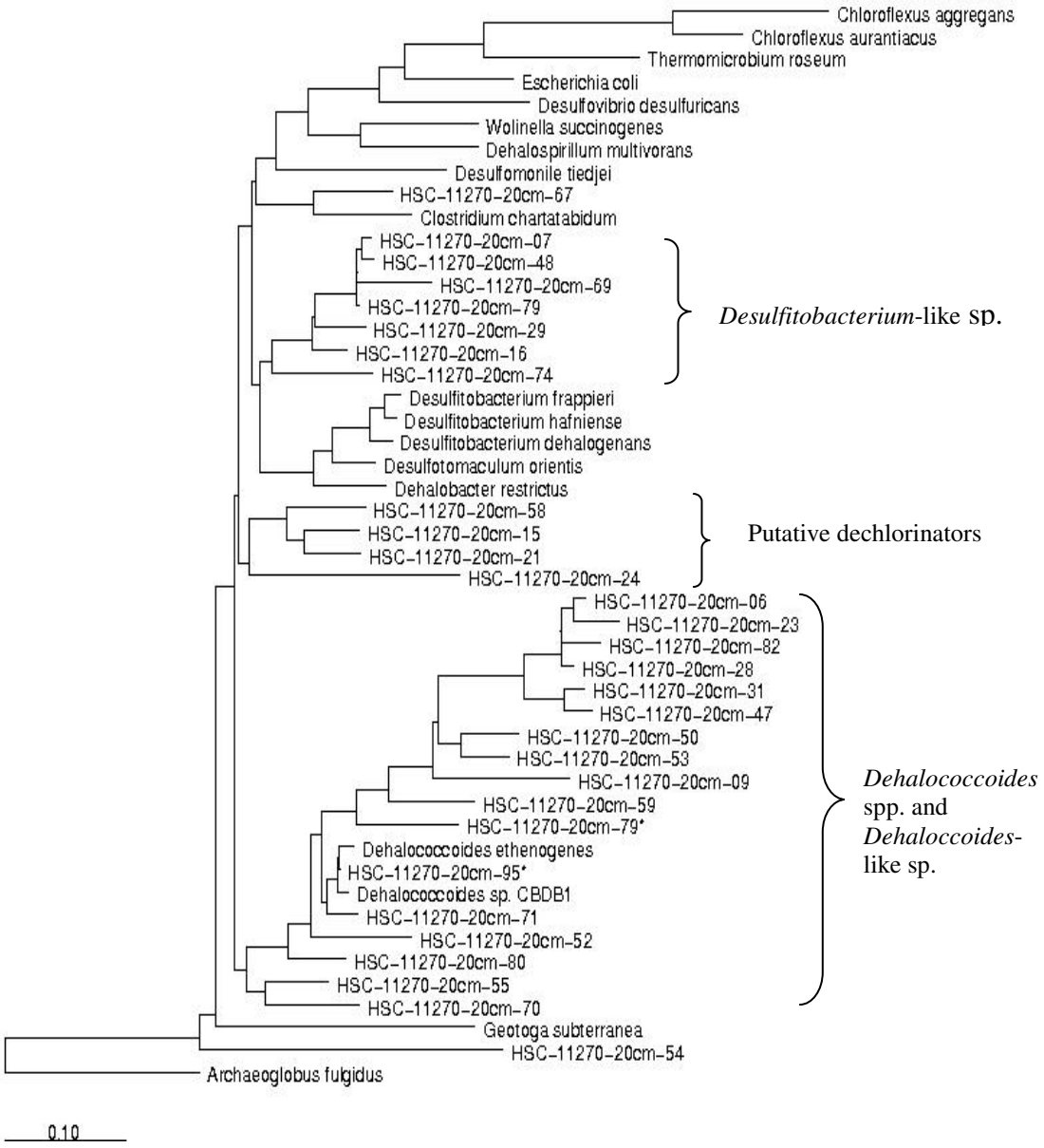

FIG. 12. Diversity of *Dehalococcoides* at 20cm (Station 11270). * indicates that bacterium was found at both depths (3cm and 20cm). The scale bar represents 10% sequence divergence.

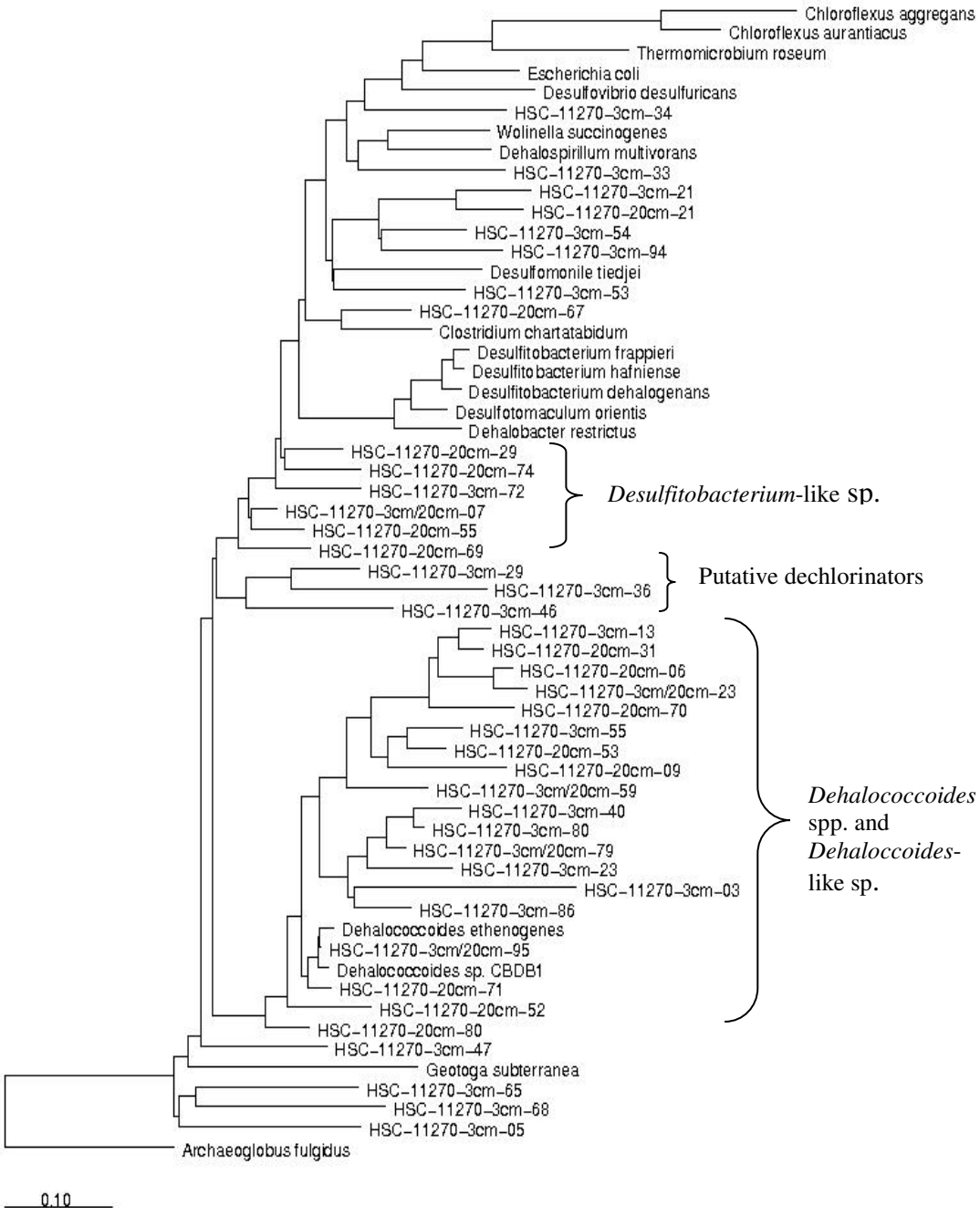

FIG. 13. Diversity of *Dehalococcoides* at 3cm and 20cm combined (Station 11270). The scale bar represents 10% sequence divergence.

Most of the clones are closely related to *Dehalococcoides*. The fact that there are some clones that belong to different phylogenetic groups is due to the primer set used to construct the 16S rRNA gene clone libraries. As discussed in the previous chapter, the primer set was not exclusively specific to *Dehalococcoides*. Several dechlorinators detected with this primer pair included the *Deltaproteobacteria* (sulfate-reducing-bacteria) *Desulfomonile*-like sp, *Desulfitobacterium*-like sp. as well as putative dechlorinators that had no identifiable closest relative. Nonetheless, the majority of the clones were members of the group *Dehalococcoides* or closely related to it. *Dehalococcoides ethenogenes* strain 195 and strain CBDB1 comprised 17.2% of the combined diversity, 18.6% at 3cm, and 15.9% at 20cm. *Dehalococcoides*-like species comprised 39.3%, 50.8%, and 28.6% of the combined, 3cm, and 20cm diversity respectively. The group *Chloroflexi*, to which *Dehalococcoides* are assumed to belong, made up 66.4% of the combined diversity. The same group comprised 62.7% and 69.8% of the 3cm and 20cm diversity respectively. 33.6% (combined depths), 37.3% (3cm), and 30.2% of all the clones belonged to groups other than *Chloroflexi* and *Dehalococcoides,* most notably the genus *Sporosarcina* (*Firmicutes*). The phylum *Firmicutes* also contains dehalorespiring groups (53). *Dehalococcoides* diversity at 20cm was greater than at 3cm. However, there does not appear to be a large difference in diversity between the two depths overall. Both depths have a cluster of clones near *Dehalococcoides ethenogenes* strain 195 and strain CBDB1, indicating that new strains may be evolving in the HSC sediments.

Dehalococcoides sp. strain CBDB1and *D. ethenogenes* strain 195 have been shown to reductively dechlorinate 1,2,3,4-TCDD (14, 15) and strain CBDB1 is also capable of dechlorinating 1,2,3,7,8-PeCDD (14). Their presence in the HSC sediments points to the dechlorination potential of not only dioxins, but also certain chlorobenzenes (15) and chlorobiphenyls (15, 63). The fact that we also found a variety of *Dehalococcoides*-like species may point to an even greater overall dechlorination potential.

For the *Dehalococcoides* clone library at 3cm, coverage was 72.58% and for the *Dehalococcoides* clone library at 20cm, coverage was 83.08%. Thus the data presented here would account for 73% (3cm) and 83% (20cm) of the clones in a similar clone library of infinite size (39). Unfortunately, the available information does not permit us to estimate the diversity of the remaining 27% (3cm) and 17% (20cm) of rDNAs that are unaccounted for. According to the Shannon's index for diversity (H'), *Dehalococcoides* diversity is higher at 3cm than it is at 20cm. H' was 1.15 and 1.12 for 3cm and 20cm respectively. I also calculated evenness (J'). J' was 0.81 and 0.85 for 3cm and 20cm respectively. This indicates that *Dehalococcoides* diversity is relatively evenly distributed. Rarefaction curves were also calculated (see Figs. 14 and 15) and show that *Dehalococcoides* diversity has been sampled fairly well. The slope is gradually flattening; indicating that only a small fraction of the *Dehalococcoides* diversity has not been sampled. It is interesting to note that almost all of the clones were similar to other non-cultured organisms from contaminated soils.

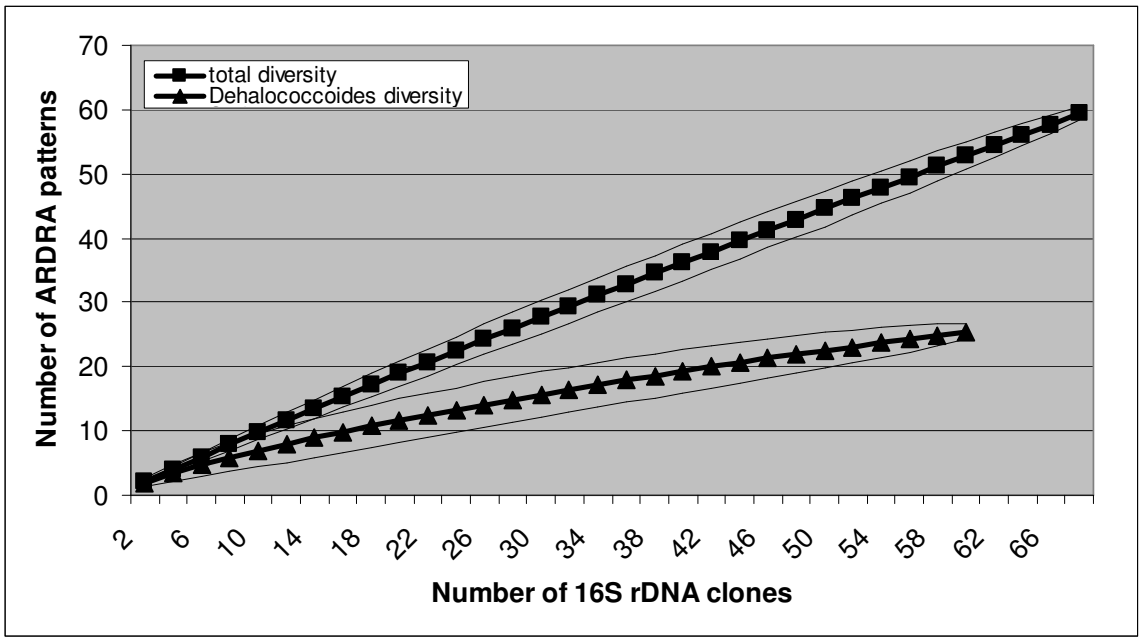

FIG. 14. Rarefaction curves for the different ARDRA patterns of 16S rDNA clones at Station 11270 at 3cm depth.

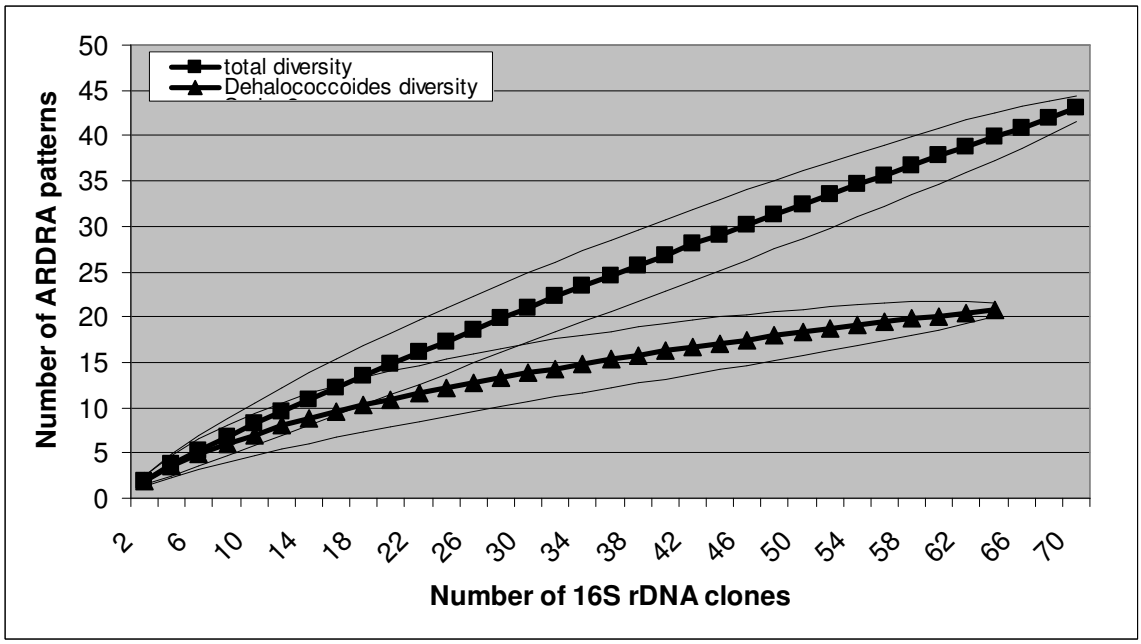

Fig. 15. Rarefaction curves for the different ARDRA patterns of 16S rDNA clones at Station 11270 at 20 cm depth.

III. 3. 2. Total Bacterial Diversity within HSC Sediments

Total bacterial diversity was characterized at one sampling location within the HSC and at two different depths. We selected Station 11270 because it had the highest dioxin concentrations out of all of the HSC sediment cores analyzed for dioxin at that time. Within this sediment core, we selected two depths for total bacterial diversity analysis. We selected 3cm since it was the first depth at which *Dehalococcoides* was detected with PCR analysis. We also chose 20cm in order to determine whether or not there was a difference in total bacterial diversity with depth and estimated age of the sediment. *Dehalococcoides* had also been detected at this depth with PCR analysis. Both of these depths were anaerobic.

Table 23 shows the results for the total bacterial diversity at 3cm, 20cm, and both. Fig. 16 shows the phylogenetic tree for the total bacterial diversity at both depths combined.

TABLE 23. Total bacterial diversity at Station 11270 at depths 3cm and 20cm.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
Betaproteobacteria				
Betaproteobacteria; Rhodocyclales; Rhodocyclaceae bacterium FTL11	96	1	1	0
Betaproteobacteria; Rhodocyclales; Rhodocyclaceae	94	1	1	0
beta proteobacterium	93	1	1	0
beta proteobacterium	94	1	1	0
Betaproteobacteria; Burkholderiales; Oxalobacteraceae;	94	1	0	1
bacterium from the Sagara petroleum reservoir (Betaproteobacteria; Burkholderiales; Oxalobacteraceae)	97	1	1	0
bacterium from a denitrifying quinoline-removal bioreactor (betaproteobacteria)	92	1	1	0
bacterium from sediments of a temperate artificial lake, Rapel reservoir (Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; Thiobacillus)	93	1	1	0
bacterium from limestone-corroding stream biofilms, frasassi cave system, Italy (betaproteobacteria)	93	1	1	0
bacterium from sulfur-oxidizing cave biofilms (betaproteobacteria)	97	1	1	0
bacterium from extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy (betaproteobacteria)	96	1	1	0
Gammaproteobacteria				
Gammaproteobacteria; Chromatiales; Chromatiaceae; Rheinheimera chironomi	99	3	3	0
Gammaproteobacteria; Alteromonadales; Alteromonadaceae; Marinobacter sp.SBS	97	1	1	0
gamma proteobacterium	97	1	1	0
Gammaproteobacteria; Oceanospirillales Neptunomonas sp. JAMM 0745	92	1	1	0
Gammaproteobacteria from hydrothermal system off Japan	96	1	1	0
gamma proteobacterium	95	1	1	0

TABLE 23. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
bacterium from a polychlorinated-dioxin-dechlorinating microbial community (gammaproteobacteria)	97	1	1	0
bacterium from sediments from the eutrophic Guanting Reservoir (Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae)	89	1	1	0
bacterium from mangrove soil (gammaproteobacteria)	98	1	1	0
bacterium from Baltic Sea sediment (gammaproteobacteria)	98	1	1	0
bacterium in ridge flank crustal fluids (Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae; Methylophaga)	96	1	1	0
Deltaproteobacteria				
Deltaproteobacterium from Gulf of Mexico gas hydrates	93	1	0	1
Deltaproteobacterium from mangrove soil	97	2	1	1
delta proteobacterium from mangrove soil	90	1	0	1
Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae	97	1	0	1
delta proteobacterium	90	1	1	0
delta proteobacterium	91	1	1	0
Deltaproteobacteria in an intertidal mud flat of the Wadden Sea	95	1	1	0
delta proteobacterium	89	1	1	0
delta proteobacterium	90	1	1	0
delta proteobacterium	93	1	1	0
bacterium from an anaerobic 1,2-dichloro propane-dechlorinating mixed culture (deltaproteobacteria)	96	1	0	1
benzene mineralizing bacterium (deltaproteobacteria)	91	1	0	1
bacterium from soils contaminated with lead, chromium and petroleum hydrocarbons (deltaproteobacteria)	91	1	1	0
bacterium from contaminated sediment (deltaproteobacteria)	96	1	1	0
bacterium from a profundal lake sediment Lake Kinnert (Israel) (deltaproteobacteria)	89	1	0	1
bacterium from a profundal lake sediment Lake Kinneret (Israel) (deltaproteobacteria)	83	1	0	1
microbe in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin (deltaproteobacteria)	93	1	0	1

TABLE 23. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
bacterium from a hypersaline mat (Deltaproteobacteria; Syntrophobacterales)	90	1	0	1
bacterium from mangrove sediment (Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae)	91	1	0	1
bacterium from mangrove soil (deltaproteobacteria)	89	1	0	1
bacterium from mangrove sediment (Deltaproteobacteria; Desulfobacterales)	90	1	1	0
bacterium from Arizona soils (Deltaproteobacteria; Desulfuromonadales; Desulfuromonadaceae)	87	1	1	0
Epsilonproteobacteria				
Epsilonproteobacteria from mangrove sediment of Xiamen, China	96	5	5	0
bacterium from a uranium-contaminated aquifer (epsilonproteobacteria)	95	1	1	0
bacterium from a hydrothermal site on the East Pacific Rise (epsilonproteobacteria)	92	1	1	0
Firmicutes				
Bacteria; Firmicutes; Bacillales; Planococcaceae; Sporosarcina sp. S11-2	97	3	0	3
similar to bacteria found in air in Austin and San Antonio (Firmicutes; Bacillales; Planococcaceae; Sporosarcina)	97	18	0	18
Firmicutes bacterium from mangrove soil	87	1	0	1
bacterium from production waters of a low-temperature biodegraded oil reservoir (Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; Fusibacter)	92	1	1	0
Chloroflexi				
Chloroflexi bacterium from mangrove soil	96	1	0	1
Chloroflexi bacterium from Lake Tanganyika	92	1	0	1
Chloroflexi bacterium	87	1	1	0
bacterium from Victoria Harbour sediment (Hong Kong) (Chloroflexi)	93	1	0	1
bacterium in a chlorinated ethene contaminated aquifer (Chloroflexi)	94	1	1	0
bacterium from methanogenic granules from a upflow anaerobic sludge bed reactor (Chloroflexi)	92	1	1	0
bacterium from sandy carbonate sediment (Chloroflexi)	94	1	0	1

TABLE 23. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
Spirochetes				
Bacteria; Spirochaetes; Spirochaetales	94	1	1	0
bacterium from sediments of Songhua River contaminated with nitrobenzene (spirochete)	95	1	0	1
bacterium from an anaerobic, trichlorobenzene-transforming microbial consortium (spirochete)	91	1	0	1
microbe in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin (spirochete)	94	2	1	1
bacterium in the Quaternary mud sediments (spirochete)	96	3	1	2
bacterium in shallow subsurface of Sarobetsu Mire (Japan) (spirochete)	93	2	1	1
Nitrospirae				
Nitrospirae bacterium	91	1	0	1
bacterium from a forested wetland impacted by reject coal (Nitrospirae)	91	1	1	0
bacterium from sediment of Guanting Reservoir (Nitrospirae)	91	2	1	1
Actinobacteria				
Actinobacteridae; Actinomycetales; from subterranean hot springs in Iceland	91	1	0	1
Planctomycetes				
Bacteria; Planctomycetes; Planctomycetacia; Planctomycetales	92	1	0	1
bacterium from Victoria Harbour sediment (Hong Kong) (planctomycete)	91	1	1	0
bacterium from contaminated sediments (planctomycete)	92	1	0	1
bacterium from sediment of Guanting Reservoir (planctomycete)	84	1	1	0
Acidobacteria				
Acidobacteria from mangrove soil	95	1	1	0
Verrucomicrobia				
Verrucomicrobia bacterium	90	2	2	0
Cytophaga				
bacterium from anaerobic reactor (Cytophaga)	92	1	0	1

TABLE 23. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
bacterium from an autotrophic denitrifying UASB reactor (Cytophaga)	95	1	1	0
Deferribacteres				
bacterium from coastal marine sediments (Deferribacteres)	85	2	0	2
Candidate Division OP1				
candidate division OP1 bacterium	88	1	0	1
Candidate Division OP3				
bacterium from contaminated sediment (Candidate Division OP3)	87	2	0	2
bacterium from deep-sea sediment (Candidate Division OP3)	81	1	0	1
Candidate Division AC1				
bacterium associated with benzoate degradation in the methanogenic consortium (Candidate Division AC1)	88	3	3	0
Candidate Division JS1				
bacterium from a mesophilic anaerobic solid waste digestor (Candidate Division JS1)	94	1	0	1
bacterium from marine sediments (Candidate Division JS1)	93	4	0	4
Unknown				
anaerobic methane-oxidizing bacterium (unknown)	95	1	1	0
bacterium from contaminated sediments (unknown)	78	1	1	0
bacterium from sediments of Lake Kastoria, Greece (unknown)	96	1	1	0
bacterium from anoxic zone of a meromictic lake (Lake Pavin) (unknown)	88	1	1	0
bacterium from the Changjiang river, Japan (unknown)	97	4	0	4
bacterium from Lake Tanganyika (unknown)	95	1	0	1
planctonic bacterium in groundwater in a deep gold mine of South Africa (unknown)	94	1	1	0
bacterium in Pearl River estuarine sediments (unknown)	92	1	1	0
bacterium from the Grotta Azzurra of Palinuro Cape (Salerno, Italy) (unknown)	92	1	0	1

TABLE 23. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones (combined depths)	Number of Clones (3 cm)	Number of Clones (20 cm)
bacterium from hypersaline Gulf of Mexico sediments (unknown)	90	1	0	1
bacterium in the Quaternary mud sediments (unknown)	87	1	0	1
bacterium in the Quaternary mud sediments (unknown)	82	1	0	1
bacterium in the Quaternary mud sediments (unknown)	93	1	1	0
bacterium from anaerobic Swine Lagoons (unknown)	78	1	0	1
bacterium from the Andean Altiplano, Chile	93	1	1	0

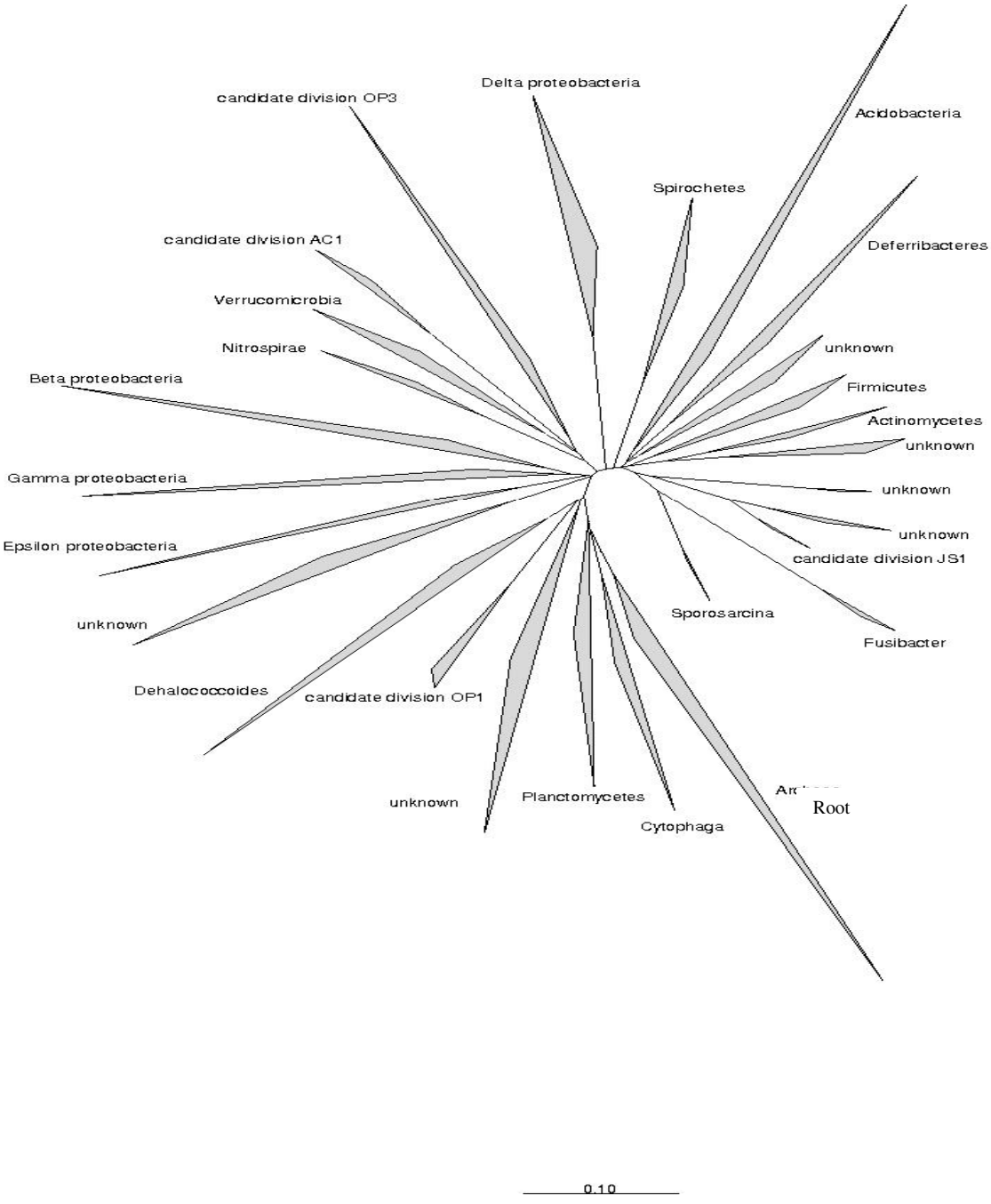

FIG.16. Total bacterial diversity at Station 11270 at depths 3cm and 20cm.

Phylogenetic Analysis of the Clone Libraries

Total bacterial diversity within HSC sediments was unexpectedly high and there was no significant difference in total bacterial diversity between depths; that is why only the phylogenetic tree displaying the combined diversity is presented here. The majority of the cloned sequence types analyzed clustered within the *Proteobacteria* (39%). *Deltaproteobacteria* was the most frequently detected lineage (16.30%), followed by *Gammaproteobacteria* (9.20%), *Betaproteobacteria* (8.50%), and *Epsilonproteobacteria* (5.00%). Another large fraction of the cloned sequence types clustered with the phylum *Firmicutes* (16.30%). *Firmicutes* are Gram-positive bacteria with a low G+C content (30). All of the phylotypes fell within two of the three classes of this phylum. The majority of phylotypes (~ 90%) clustered with *Bacilli*, in particular with the genus *Sporosarcina*. These phylotypes were most closely related to uncultured bacteria detected in urban aerosols from San Antonio and Austin, TX (Brodie et al, 2007). Since *Sporosarcina* are endospore formers it is not too surprising to find these bacteria in air as well as sediment. The remaining 10% of the phylotypes that clustered with the *Firmicutes* fell within the class *Clostridia*. 7.10% of the phylotypes clustered within the phylum *Spirochetes*. Another 5.00% of the phylotypes fell within *Chloroflexi*, which contains *Dehalococcoides*. A relatively small number of clones (10.5%) clustered with the phyla *Nitrospira* (2.80%), *Planctomycetes* (2.80%), *Verrucomicrobia* (1.40%), *Cytophaga* (1.40%), *Deferribacteres* (1.40%), and *Holophaga/Acidobacteria* (0.70%). 8.4% of the cloned sequence types clustered with various candidate divisions. Of the 8.4% of clones, 3.5% fell within JS1, 2.10% within AC1, 2.10% within OP3, and 0.70%

within OP1. The remaining 12.80% of cloned sequence types did not have a known closest relative. These clones were most closely related to other uncultured bacteria from various (contaminated) sites around the world including a meromictic lake, the Changjiang River (Japan), groundwater in a deep gold mine in South Africa, caves in Italy, hypersaline sediments from the Gulf of Mexico, the Andean Altiplano (Chile), and anaerobic Swine Lagoons.

Coverage, Diversity, and Rarefaction Analysis

For the total bacteria clone library at 3cm, coverage was 18.57% and for the total bacteria clone library at 20cm, coverage was 48.61%. Thus the data presented here would account for 19% (3cm) and 47% (20cm) of the clones in a similar clone library of infinite size (39). Unfortunately, the available information does not permit us to estimate the diversity of the remaining 81% (3cm) and 53% (20cm) of rRNAs that are unaccounted for. These numbers are significantly lower than the ones for *Dehalococcoides* diversity and show that total bacterial diversity was extremely high. For each of these two total bacteria clone libraries approximately 70-80 clones were analyzed. As the numbers indicate, a much larger clone library is needed, especially in the upper centimeters to accurately depict total bacterial diversity. However, these results are promising when put in the context of bioremediation, meaning that such a high bacterial diversity is likely to support many different groups of dehalorespiring bacteria.

According the Shannon's index for diversity (H'), total bacterial diversity is higher at 3cm than it is at 20cm. H' was 1.75 and 1.43 for 3cm and 20cm respectively. I also calculated evenness (J'). J' was 0.98 and 0.87 for 3cm and 20cm respectively. This indicates that total bacterial diversity is pretty evenly distributed. Both H' and J' are higher than for *Dehalococcoides* diversity. This is to be expected since *Dehalococcoides* only makes up part of the overall bacterial diversity. Rarefaction curves were also calculated (Figs. 14 and 15) and show that overall bacterial diversity has not been sampled well. The slope is steep and not flattening at all indicating that only a small fraction of the overall bacterial diversity has been sampled. Again, it is interesting to note that almost all of the clones were similar to other non-cultured organisms from (contaminated) soils.

III. 3. 3. Total Bacterial Diversity within Wetland Control Sediments

Total bacterial diversity at a wetland control site was examined to better understand the differences in bacterial diversity within HSC (high dioxin concentrations) sediments and 'pristine' sediments (low dioxin concentrations). We constructed a 16S rRNA gene clone library at 20cm for Station FW1A since 20cm was also a depth chosen for the previous clone libraries.

Table 24 shows the results for the total bacterial diversity at 20cm for Station FW1A. Fig. 17 shows the phylogenetic tree for the total bacterial diversity 20cm for Station FW1A.

TABLE 24. Total bacterial diversity at Station FW1A at depth 20cm.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones	Number of Clones in Group
Alphaproteobacteria			
Uncultured Hyphomicrobium sp. (alphaproteobacteria)	98	1	
Sphingomonas sp. K101 (Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas)	98	2	
Uncultured bacterium clone FCPO401 (alphaproteobacteria)	98	2	
Uncultured proteobacterium clone DOK_NOFERT_clone587 (probably alpha)	93	1	
			6
Betaproteobacteria			
Macromonas bipunctata (Betaproteobacteria; Burkholderiales; Comamonadaceae; Macromonas)	97	1	
Uncultured bacterium clone Amb_16S_1203 (Betaproteobacteria; Burkholderiales; Oxalobacteraceae)	98	1	
Janthinobacterium sp. A1-13 (Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Janthinobacterium)	97	1	
Uncultured bacterium (Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Duganella)	98	1	
Duganella sp. BD-a14 (Betaproteobacteria; Burkholderiales;Oxalobacteraceae;Duganella)	96	1	
Uncultured bacterium clone:BSN41 (Betaproteobacteria; Burkholderiales; Oxalobacteraceae; Herbaspirillum)	99	1	
Cupriavidus basilensis (Betaproteobacteria; Burkholderiales;Burkholderiaceae;Cupriavidus)	98	2	
Uncultured Aquabacterium sp. clone C-23 (Betaproteobacteria; Burkholderiales; Aquabacterium)	99	1	
Ralstonia sp. HI3 (Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia)	99	1	
Mercury-resistant bacterium mCFU 581 (Betaproteobacteria; Neisseriales; Neisseriaceae)	98	2	
Uncultured bacterium clone JH-WHS153 (betaproteobacteria)	98	1	
Uncultured bacterium clone F1E (betaproteobacteria)	99	4	
Uncultured bacterium clone aab20a10 (betaproteobacteria)	94	1	
Uncultured bacterium isolate High.2.45.F10.HB35 (betaproteobacteria)	99	1	
Uncultured proteobacterium clone Amb_16S_1080 (betaproteobacteria)	98	3	
Uncultured hydrocarbon seep bacterium BPC087 (betaproteobacteria)	97	1	
Beta proteobacterium G5G6 (betaproteobacteria)	97	4	
			27
Gammaproteobacteria			
Uncultured proteobacterium clone GASP-WDOW3_D09 (Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Psychrobacter)	96	1	

TABLE 24. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones	Number of Clones in Group
Escherichia coli (Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Escherichia)	97	3	
			4
Deltaproteobacteria			
Uncultured bacterium clone WIT-Mm-5 (deltaproteobacteria)	94	1	
Uncultured bacterium clone D28213 (Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter; Fe(III)-reducer)	95	2	
Uncultured bacterium clone FFCH6412 (Deltaproteobacteria; Myxococcales; Cystobacterineae; Myxococcaceae; Anaeromyxobacter)	97	1	
Uncultured delta proteobacterium clone JAB NFA 300	98	1	
Uncultured proteobacterium clone Amb_16S_816 (probably delta)	95	1	
Uncultured bacterium MERTZ_2CM_350 (proteobacteria, probably delta)	84	1	
			7
Acidobacteria			
Uncultured bacterium clone JH-WHS179 (Acidobacteria; Acidobacteriales; Acidobacteriaceae;Holophaga)	95	3	
Uncultured bacterium clone JH-WHS24 (Acidobacteria; Acidobacteriales; Acidobacteriaceae; Holophaga)	93	2	
Uncultured Acidobacteriaceae bacterium clone Amb_16S_1232 (Acidobacteria; Acidobacteriales; Acidobacteriaceae)	97	2	
Uncultured Acidobacteriaceae bacterium clone Amb_16S_825 (Acidobacteria; Acidobacteriales; Acidobacteriaceae)	97	1	
Uncultured bacterium DA038 (Acidobacteria; Acidobacteriales; Acidobacteriaceae)	96	1	
Uncultured bacterium clone Elev_16S_1241 (Acidobacteria)	97	1	
Uncultured bacterium clone NR.1.031 (Acidobacteria)	98	1	
Uncultured bacterium clone Elev_16S_1445 (Acidobacteria)	97	5	
Uncultured bacterium clone JH-WH227 (Acidobacteria)	96	1	
Uncultured bacterium clone 116 (Acidobacteria)	93	1	
Uncultured bacterium clone FAC72 (Acidobacteria)	96	1	
Uncultured bacterium clone GASP-77KA-695-G03 (Acidobacteria)	97	1	
Uncultured bacterium clone AH33 (Acidobacteria)	97	1	
Uncultured bacterium clone FCPT625 (Acidobacteria)	99	3	
Uncultured bacterium clone 656042 (Acidobacteria)	93	1	
Uncultured bacterium clone JH-WHS68 (Acidobacteria)	97	2	
Uncultured bacterium clone 1894a-22 (Acidobacteria)	95	1	
Uncultured soil bacterium clone UH8 (probably Acidobacteria)	98	1	
Uncultured bacterium clone FCPT535 (Acidobacteria?)	96	1	

TABLE 24. Continued.

Closest 16S rRNA relative in GenBank and ARB database	% Similarity	Number of Clones	Number of Clones in Group
Uncultured bacterium clone Amb_16S_1669 (probably Acidobacteria)	98	3	
			33
Firmicutes			
Uncultured organism clone ctg_CGOGA60 (Firmicutes; Bacillales; Bacillaceae; Bacillus)	99	1	
Uncultured Bacillus sp. clone EHFS1_S09a (Firmicutes; Bacillales; Bacillaceae; Bacillus)	98	1	
Uncultured organism clone ctg_CGOGA51 (Firmicutes; Bacillales; Bacillaceae)	98	1	
Uncultured bacterium clone JH-WHS189 (Firmicutes)	97	1	
Uncultured Firmicutes bacterium clone GASP-KC2W2_E10	96	1	
			5
Verrucomicrobia			
Uncultured bacterium clone JH-WHS202 (Verrucomicrobia)	98	1	
Uncultured bacterium clone JH-WH40 (Verrucomicrobia)	99	1	
Uncultured bacterium clone JH-WHS173 (Verrucomicrobia)	97	1	
			3
Planctomycetes			
Uncultured bacterium clone FAC47 (Planctomycetes; Planctomycetacia)	98	1	
Uncultured bacterium clone B16 (planctomycete)	92	1	
			2
Actinobacteria			
Uncultured actinobacterium clone EB1077	98	1	
Uncultured soil bacterium clone 1215-2 (probably actinobacteria)	98	1	
			2
Nitrospirae			
Uncultured Nitrospirae bacterium clone BB54 (Nitrospirae)	97	2	
			2
Gemmatimonadetes			
Uncultured bacterium clone 2A-8 (Bacteria; Gemmatimonadetes)	97	1	
			1
Candidate Division SPAM			
Uncultured bacterium clone CV52 (Bacteria; candidate division SPAM)	97	1	
			1

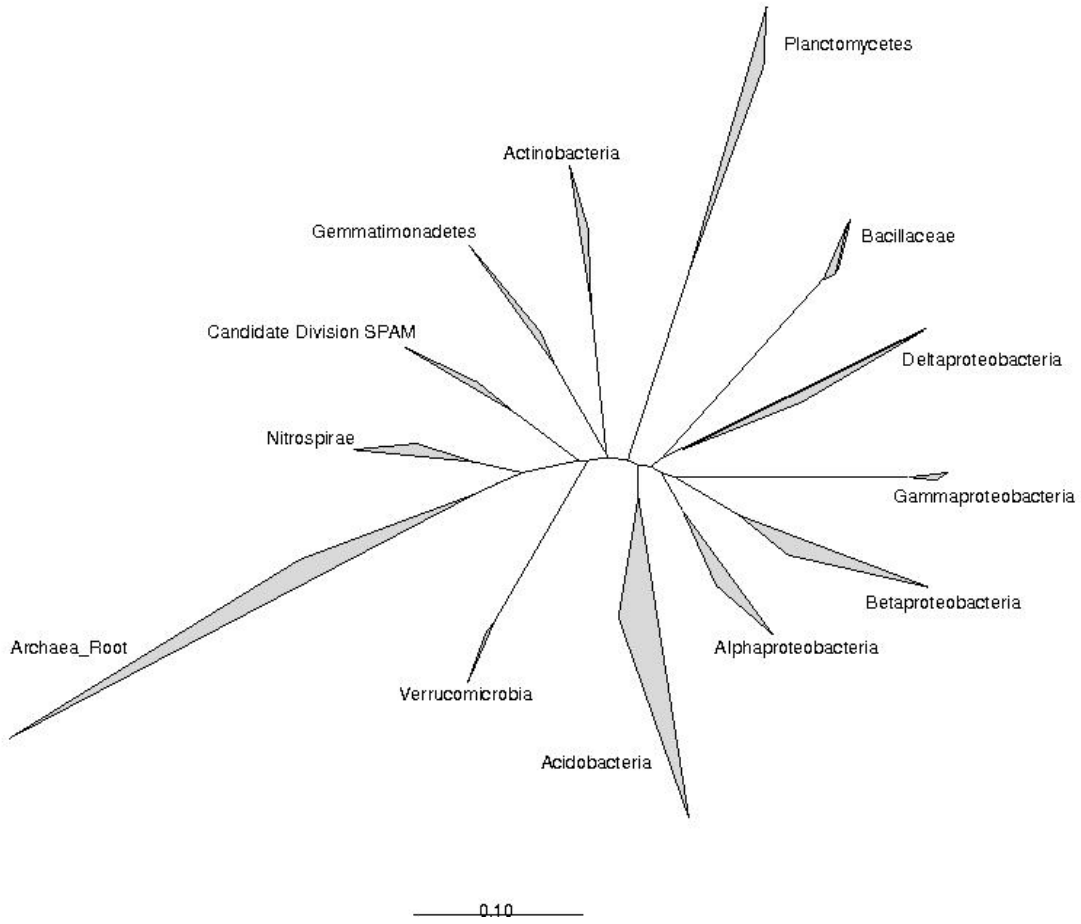

Fig. 17: Total bacterial diversity at the wetlands control site FW1A at 20cm.

Phylogenetic Analysis of the Wetlands Control Clone Library

Total bacterial diversity at the wetlands control site is significantly less than compared to the total bacterial diversity observed in the HSC sediments. Almost half of the cloned sequence types analyzed clustered within the *Proteobacteria* (47%), compared to 39% in the HSC sediments. *Betaproteobacteria* was the most frequently detected lineage (29.00%), followed by *Deltaproteobacteria* (7.50%), *Alphaproteobacteria* (6.50%), and *Gammaproteobacteria* (4.30%). *Epsilonproteobacteria* was absent in the wetlands

control sediments, however a small fraction (5.00%) had been detected in the HSC sediments. On the other hand, *Alphaproteobacteria* was absent in the HSC sediments, but present in the wetlands control sediments. *Deltaproteobacteria* in the wetlands control sediments only comprised 7.50%, compared to 16.3% in the HSC sediments. Within the *Proteobacteria*, dominance shifted from *Deltaproteobacteria* (in the HSC sediments) to *Betaproteobacteria* (wetlands control sediments). Most of the sequence types that grouped within the *Betaproteobacteria* were related to sequences of species within the order *Burkholderiales* (10 sequence types out of 27). Furthermore, the cloned sequences clustered within the families *Oxalobacteraceae*, *Burkholderiaceae*, and *Comamonadaceae*. As shown in Table 24, the cloned sequences were highly similar (97-99% similarity) to the 16S rRNA gene sequences of *Janthinobacterium* spp., *Duganella* spp., *Herbaspirillium* spp. (nitrogen-fixing bacteria), *Cupriavidus* spp., *Ralstonia* spp. (hydrogen-oxidizing bacteria), *Macromonas* spp. (metabolizes organic acids), and *Aquabacterium* spp. The second most abundant group in the clone library clustered within the phylum *Holophaga/Acidobacteria* (35.50%). Clone sequences in this group were similar to other uncultured *Acidobacterium*-related cloned 16S rRNA gene sequences retrieved from different soils, i.e. cloned sequences from an iron manganese nodule (GenBank accession number EF492958) and cloned sequences from soil associated with Trembling Aspen, a deciduous tree (GenBank accession number EF18770). The third most abundant group in the clone library clustered within the phylum *Firmicutes* (5.40%). The majority of the phylotypes (3 out of 5) fell within the class *Bacilli*, in particular within the genus *Bacillus*. The remaining cloned sequence

types clustered with the phyla *Verrucomicrobia* (3.20%), *Planctomycetes* (2.20%), *Actinobacterium* (2.20%), *Nitrospira* (2.20%), and *Gemmatimonadetes* (1.10%). 1.10% of the cloned sequence types clustered with the candidate division SPAM. This clone was similar to uncultured bacteria found on extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy (Macalady et al, 2007).

Coverage, Diversity, and Rarefaction Analysis

Coverage for this clone library was 44.44%. Thus the data presented here would account for 44% of the clones in a similar clone library of infinite size (39). Unfortunately, the available information does not permit us to estimate the diversity of the remaining 56% (20cm) of rRNAs that are unaccounted for. These numbers are similar to the total bacteria clone libraries from the HSC. For this clone library 108 clones were analyzed. This is an increase of about 30 clones compared to the other two clone libraries from the HSC. According the Shannon's index for diversity (H'), total bacterial diversity was 1.83. This value is slightly higher than for the other two clone libraries (3cm-1.75 and 20cm-1.43). Even though the phylogenetic analysis revealed that there were fewer bacterial groups, individual clone diversity was still high. This could also be due to the fact that about 30 more clones were analyzed for the wetlands control clone library than for the two HSC clone libraries. Since the coverage calculations showed that only a small portion of the diversity was analyzed for the HSC clone libraries, it is expected that diversity will increase with more clones examined. Evenness, J', was 0.97. This indicates that total bacterial diversity is pretty evenly distributed. Both H' and J' are

higher than for total bacterial diversity within the HSC. A rarefaction curve was also calculated (Fig. 18) and shows that overall bacterial diversity has not been sampled well; however, it has been sampled more completely than for the two clone libraries from the HSC. The slope is steep and slightly flattening towards the end indicating that overall bacterial diversity is being approached. Again, it is interesting to note that almost all of the clones were similar to other non-cultured organisms from soils around the world.

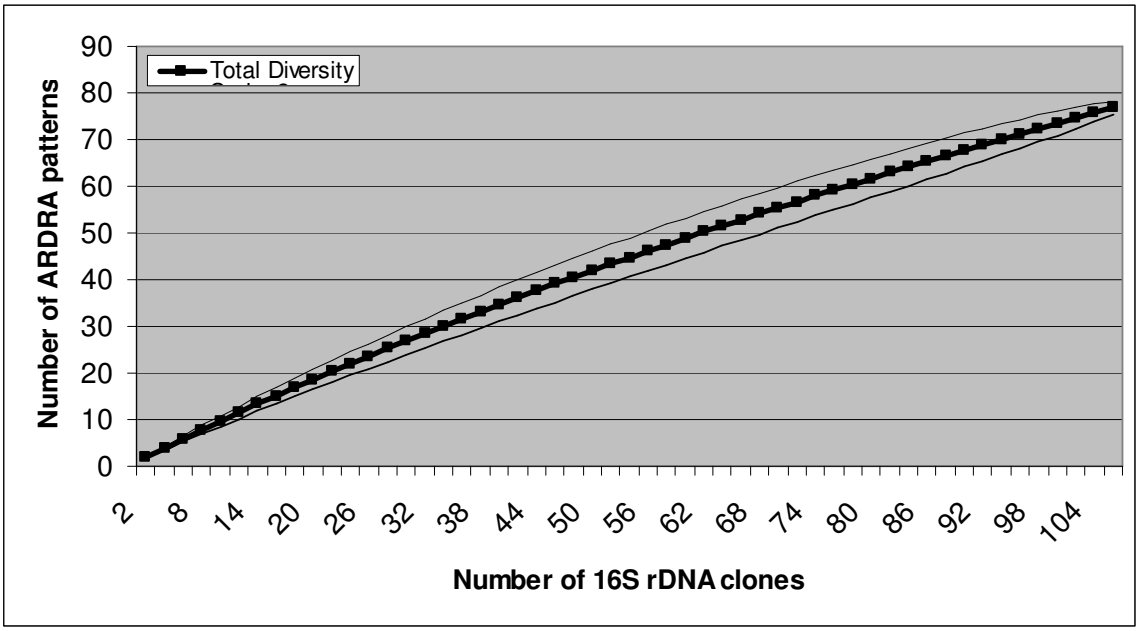

FIG. 18. Rarefaction curve for the different ARDRA patterns of 16S rDNA clones for the wetlands control site FW1A.

Since diversity is still not sampled well when looking at individual clones, I investigated whether or not diversity is better sampled when considering phylogenetic groups. I calculated coverage, diversity, evenness, and a rarefaction curve for the wetlands control clone library considering 13 phylogenetic groups. Coverage for the groups was 76.92%.

This is a dramatic increase compared to 44.44% for individual clones. This indicates that the phylogenetic groups are well covered within the sediment, but not their individual members. According the Shannon's index for diversity (H'), total bacterial diversity was 0.81. This is significantly lower than 1.83 for individual clones. Evenness (J') was 0.73. This value is also lower compared to 0.97 for individual clones. This evenness may actually represent the diversity a little better, since phylogenetic analysis showed that two groups were dominant. The rarefaction curve is also flattening, indicating that when considering phylogenetic groups, diversity is well sampled by this clone library (Fig. 19).

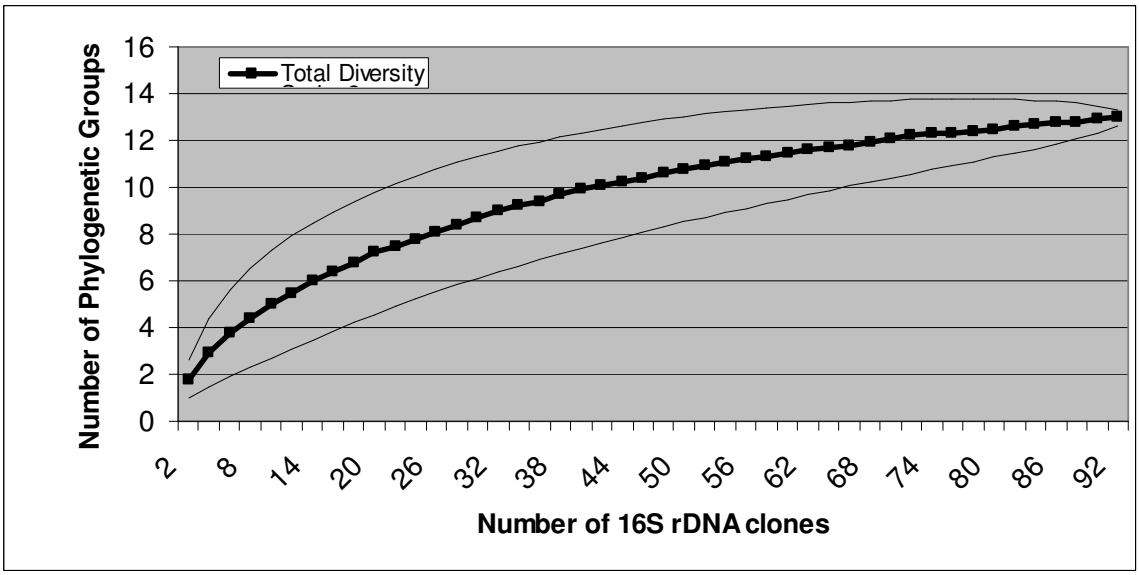

FIG. 19. Rarefaction curve for the different phylogenetic groups of the 16S rDNA clones for the wetlands control site FW1A.

III. 3. 4. Conclusions

Sediments in the HSC contained a high total bacterial diversity as well as a diverse population of *Dehalococcoides spp.*, which dehalogenate various toxic substances, including dioxin. *16S rRNA clone libraries targeting Dehalococcoides spp. was dominated* (~17%, combined depths) *by* two strains, known to dehalorespire dioxins, *Dehalococcoides* sp. strain CBDB1and *Dehalococcoides ethenogenes* strain 195. *Dehalococcoides*-like species comprised between 29% (20cm) and 51% (3cm) of the overall *Dehalococcoides* diversity, indicating that new strains may be evolving in response to the high concentrations and diverse inputs of dioxins and other halogentated compounds in the HSC. The HSC predominant strains, *Dehalococcoides* sp. strain CBDB1and *D. ethenogenes* strain 195, have been shown to reductively dechlorinate 1,2,3,4-TCDD (14, 15) and strain CBDB1 is also capable of dechlorinating 1,2,3,7,8-PeCDD (14). Their presence in the HSC sediments points to the dechlorination potential of not only dioxins, but also certain chlorobenzenes (15) and chlorobiphenyls (15, 63). The high diversity of *Dehalococcoides*-like species may point to an even greater overall dechlorination potential. It has been speculated that anthropogenic compounds select for microbes that have acquired the ability to use them (53). The diversification of reductive dehalogenase functions in *D. ethenogenes* 195 appears to have been mediated by recent genetic exchange and amplification (53). This leads to the conclusion that the *Dehalococcoides* population is adapting to its surrounding energy sources and that through genetic exchange, more species will be able to dechlorinate highly chlorinated

compounds, such as dioxins, PCBs, and chlorobenzenes. The HSC sediments may very well be the next "breeding ground" for new *Dehalococcoides* strains.

Higher diversity of *Dehalococcoides*-like species was found at 3cm than at 20cm and may be a contributed to fresher POC content in sub-surface layers however our analysis of POC showed no difference in concentrations of POCs throughout the cores. There is also the question of origin of anaerobic Dehalococcoides in the upper sediment layers. Perhaps they are deposited from the water column and migrate deeper into the sediments. However this has not yet been investigated.

Total bacterial diversity is extremely high within HSC sediments compared to the control site FW1A. This explosion in diversity may be due to the high level of contamination in the HSC sediments. It seems that bacteria from many different phylogenetic groups, especially *Deltaproteobacteria*, *Dehalococcoides*, and *Firmicutes*, thrive on a wide variety of available substrates. Almost all of the cloned sequences from the HSC sediments were most similar to other uncultured bacteria rather than to known cultivated isolates indicating the evolutionary complexity of this environment. In the HSC sediments *Proteobacteria*, especially *Deltaproteobacteria*, were dominant. They were followed in dominance by *Firmicutes* (16.30%). Both *Deltaproteobacteria* and *Firmicutes* are known to have members that are capable of dehalorespiration (53). The *Chloroflexi clade*, which is closely related to *Dehalococcoides* or is even thought to include *Dehalococcoides,* only represented 5% of the total bacterial diversity. Low

coverage of the clone libraries due to poor PCR primer specificity may be the cause, since this clade usually predominates PCB and dioxin contaminated sediments. Difficulties in detecting *Dehalococcoides* in sediments, even laboratory sediment cultures, are not unusual when using bacterial specific primers (63). After confirming the presence of a *Dehalococcoides*-like population in a sediment culture via DGGE (denaturing gradient gel electrophoresis), Yan et al (63) constructed a 16S rRNA clone library which failed to produce sequences that corresponded to the *Dehalococcoides*-like population. After generating a second clone library with Dehalococcoides specific, the correspondence between the *Dehalococcoides*-like DGGE bands and the *Dehalococcoides*-like clones was established based on a perfect and exclusive match.

Total bacterial diversity at the wetlands control site (FW1A) was significantly lower than that observed in the HSC sediments. Almost half of the cloned sequence types detected clustered within the *Proteobacteria* (47%), as compared with 39% in the HSC sediments. The *Betaproteobacteria,* which typically dominate freshwater environments (34), were predominant (29.00%), compared to the *Deltaproteobacteria* (16.3%) in the HSC sediments. The second most abundant group in the FW1A clone library clustered within the phylum *Holophaga/Acidobacteria* (35.50%). There are fewer phylogenetic groups in the wetlands control site, however, diversity based on individual clones was higher indicating functional specialization within each group. Alternatively, it may be an artifact of a larger sampling size for the wetlands control clone library.

Overall, the results of this study are very promising for (*in situ*) bioremediation in the HSC, since it appears there is a wide variety of bacterial groups present which are able to utilize toxic substances. To my knowledge, this study is the first to examine diversity of dechlorinating bacteria, in particular *Dehalococcoides*, in natural estuarine sediments (not microcosms). In the dioxin contaminated sediments of the HSC, *Dehalococcoides* and *Dehalococcoides*-like bacteria were detected; however, none were detected in the wetlands control sediment. This confirms that *Dehalococcoides* require dioxins and other polychlorinated compounds as their terminal electron acceptors, i.e. dehalorespiration. This study also confirms that PCR detection of *Dehalococcoides* using simple 'present/not present' results is a powerful tool to determine contamination of sediments with dioxin or other polychlorinated compounds.

CHAPTER IV

DIVERSITY OF ACTIVE AND NON-ACTIVE MEMBERS OF THE MICROBIAL COMMUNITY IN SEDIMENTS FROM THE HOUSTON SHIP CHANNEL

IV. 1. Introduction

IV. 1. 1. Houston Ship Channel

The Houston Ship Channel (HSC), located in the San Jacinto River Basin, in the northwest corner of Galveston Bay, Texas, is 50 miles in length, extending from the Port of Houston to the Gulf of Mexico (Ch. I, Fig. 1). The Port of Houston is the sixth largest seaport in the world and handles more foreign water-borne tonnage than any other U.S. port. The Port of Houston generates over $10 billion annually and each year more than 6,300 vessels pass through the HSC. The HSC is also home to the largest petrochemical complex in the United States and the second largest worldwide (43). The HSC is continually being dredged and the dredged sediment is used to create spoil islands and wetlands. Both the HSC and upper Galveston Bay (GB) are highly polluted with dioxins, dioxin-like compounds, and many other contaminants, such as hydrocarbons, from industrial and municipal effluents and runoff, as well as from atmospheric wet and dry deposition. In 1990, dioxins were detected in fish and crab tissue obtained from the HSC. A seafood consumption advisory for catfish and blue crabs was issued for the HSC and upper GB, and remains in effect to this day. Subsequently, the HSC was placed on the §303 (d) list of impaired water bodies as required by the 1977 Clean Water Act (as amended, 1996) and a total maximum daily loads (TMDL) study was initiated by the

Texas Commission on Environmental Quality (TCEQ). The study revealed that Toxic Equivalent (TEQ) concentrations in water ranged from 0.10 to 3.16 pg TEQ/L and in bottom sediments from 0.9 to 139.8 ng/kg dry wt. (57). On average, dioxin concentrations exceeded the Texas Surface Water Quality Standard (0.093 pg/L) in more than 80% of all samples (48). The study also revealed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the major contributor to total TEQs in all samples. The entire HSC is contaminated with dioxins and recent dioxin inputs to the HSC continue despite regulatory efforts (64).

IV. 1. 2. Dioxins

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), commonly referred to as dioxins, are persistent environmental contaminants. Dioxins cause a variety of biochemical, immunological, and reproductive effects in animals and are suspected carcinogens (6, 7, 28, 42, 46, 51). Dioxins bioaccumulate in the aquatic and terrestrial food chains posing significant and persistent risks to human health. The estimated half-life of dioxin in the human body is 7-8 years (58). Primary sources of dioxins include the production of herbicides (56), paper and pulp bleaching, metal smelting, and waste incineration (16, 18, 54, 62). Dioxins and dioxin-like compounds are hydrophobic and therefore have a high particle and lipid affinity. Their water solubility is estimated to be 19.3 ng/L (58). Due to their high hydrophobicity, dioxins present in the water column rapidly partition to organic carbon fractions (i.e black carbon) in suspended soils and can subsequently be buried in sediments (11, 38,

52). Re-suspension of polluted sediments may re-introduce dioxins into the aquatic food chain; however, this process has not been thoroughly investigated. From both fiscal and environmental perspectives, in situ microbial remediation of dioxins in the HSC and GB is preferable to alternatives, such as removal of contaminated sediments to landfills or chemical treatments. Microbial remediation would also not interfere with the vessel traffic through the HSC. Since the HSC is tidally influenced, dioxin contamination has been transferred up- and downstream of the channel, increasing the urgency of remediation.

IV. 1. 3. Microbial Dechlorination

Studies of microbial dechlorination of polychlorinated compounds have been mostly limited to freshwater systems and have indicated that degradation rates are enhanced under anaerobic, reducing conditions (1). Quensen et al. (44) showed that the chlorinated compound DDE (1,1-dichloro-2,2,-bis(p-chlorophenyl)ethylene), a commercial by-product in DDT formulations, is preferentially degraded under methanogenic and sulfidogenic conditions. Another study found that 2,3,7,8-TCDD was degraded up to 86% under anaerobic, reducing conditions (25) (Ch. I; Fig. 2). The reductive dehalogenation of chlorinated aromatic compounds has been identified as an energy-yielding process in a number of anaerobic bacteria (22). These anaerobic bacteria use polychlorinated compounds as electron acceptors and hydrogen as an electron donor (2, 14, 22). The reductively dechlorinating bacteria known to date belong to the low GC Gram-positive bacteria (*Desulfitobacterium* and *Dehalobacter*) or to the Proteobacteria

(for example, *Desulfomonile*, *Desulfuromonas* and *Dehalospirillum*) (22, 33). Another bacterial group, *Dehalococcoides*, is also known to reductively dechlorinate highly chlorinated compounds, making the resulting congeners and other biproducts more susceptible to degradation by other bacterial groups. The closest phylogenetic affiliation of *Dehalococcoides* is with the green non-sulfur bacteria (20, 24, 60), however there is increasing evidence that they may constitute a new division of bacteria (22, 33). Thus far, *Dehalococcoides* have only been isolated from groundwater and other freshwater systems. *Dehalococcoides ethenogenes* strain 195, isolated from contaminated groundwater, is the only known isolated organism capable of fully dechlorinating tetrachloroethene (PCE) and other chloroethenes to the non-toxic end-product ethene (2). Strains FL2 (isolated from a highly enriched PCE-to-ethene dechlorinating mixed culture from Red Cedar River sediment, Michigan, Loeffler et al, 2000) and DCEH2 (isolated from a dechlorinating enrichment mixed culture, GenBank accession number AJ249262) also dechlorinate chloroethenes (20). Strain CBDB1 (isolated from an enriched chlorobenzene-dechlorinating mixed culture from Saale River sediment, Germany) dechlorinates trichlorobenzenes and tetrachlorobenzenes to dichlorobenzenes, but is unable to dechlorinate PCE or trichloroethene (2). Strain CBDB1 is also able to dechlorinate chlorinated benzenes (14). Members of *Dehalococcoides* have also been shown to dechlorinate commercial polychlorinated biphenyls (PCBs) (i.e. Aroclor 1260) (9). Bedard (9) also found that *Dehalococcoides* obtain energy for growth from dechlorination. In 2003, Bunge et al (9) showed that strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (1,2,3,4-TCDD) and

1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD). Since the dehalogenation of dioxin by *Dehalococcoides* is orders of magnitude faster (weeks versus 1 to 4 years) than its anaerobic co-metabolic reduction (1, 14) its presence in or addition to dioxin contaminated areas is a significant contribution for bioremediation (1, 2, 14, 22).

Despite the many advances made in enhancing bioremediation at off-site facilities, *in situ* bioremediation is still a growing technology. Most of our knowledge about dioxin degradation comes from studies of cultivated strains of dechlorinating bacteria (2, 14, 9, 15). Estimations of rates of *in situ* dioxin degradation require a determination of the most effective dechlorinators under different conditions (i.e. pH, redox, carbon sources, temperature, & salinity), their distribution, and their densities at a contaminated site. One objective of the Sea Grant funded project was to examine the rates of 2,3,7,8-TCDD degradation in microcosm incubations after addition of different forms of carbon substrates (i.e. pyruvate, acetate and glycerol). Another objective of the Sea Grant funded project was to determine the amount of particulate organic carbon as well as several chemical constituents that influence redox zonation. In Chapter II, I demonstrated the extensive density and distribution of *Dehalococcoides* spp. bacteria in the HSC. Equally important to estimate rates of dioxin degradation is knowledge of the metabolically active fraction of the dechlorinating bacterial community. Traditionally, studies of bacterial community diversity have examined genomic DNA that encodes for 16S rRNA. Increasing numbers of studies are demonstrating the importance of comparing the 16S rRNA fraction to the 16S rDNA fraction of total nucleic acids to

better interpret the importance of individual bacterial community members to substrate utilization (36, 41, 35, 55, 5, Dar et al. 2007; 31).

IV. 1. 4. Objective

I compared the metabolically active members of the bacterial community (i.e. producing ribosomes) at the time of sampling to those that may be present but in a resting state. To accomplish this objective, I constructed 16S rRNA clone libraries from the genomic DNA that encodes for ribosomal RNA (rDNA clones) and ribosomal RNA (rcDNA clones) present in the ribosomes. To differentiate the most active fraction(s) of the microbial community from those bacteria which are present, total community RNA was reverse transcribed into complementary DNA (cDNA) and then amplified with PCR and bacterial specific primers.

> *Hypothesis 4:* I expect dechlorinating bacteria to be among the active members of the bacterial community.

I tested this hypothesis by constructing 16S rRNA gene clone libraries from both RNA (rcDNA) and DNA (rDNA). Total RNA and DNA was extracted from the same sample and 16S rRNA gene clone libraries were constructed and analyzed with restriction enzyme digests. Clones were sequenced to determine bacterial diversity. Differences in diversity of these clone libraries was examined to determine the metabolically active members of the bacterial community.

IV. 2. Materials and Methods

IV. 2. 1. Sampling

Shorter sediment cores (~30cm) for RNA analysis were collected at selected sites along the HSC (Ch. I; Fig. 3 and Table 1). RNA sediment cores were sectioned at 1cm intervals and aliquots were collected in the same manner as described before. Aliquots were taken within 24 hours of sediment core collection and stored at -80°C until later analysis.

For this part of my thesis, certain sediment samples were selected for the determination of active and inactive members of the microbial community in the HSC. In order to determine the active and inactive members of the microbial community in HSC sediments, we selected Station SG-6 because it was located near a historic paper mill and Suarez et al (57) showed that it had high dioxin concentrations. Within this sediment core, we selected one depth for bacterial diversity analysis. We selected 15cm since it was comparable to other depths used in this study.

IV. 2. 2. RNA/DNA Extraction

Total RNA and DNA from environmental sediment samples were extracted using the RNA Power Soil Total RNA Isolation Kit (MoBio). Frozen sediment samples were thawed on ice for no more than 30 minutes and 1g of sediment was removed and placed in the first MoBio kit conical tube. Nucleic acids were extracted according to the manufacturer's protocol. Best RNA laboratory technique was applied to every extent

possible. Following nucleic acid extraction, the DNA samples were stored at -20°C for later analysis. A PCR reaction with the bacterium-specific primers 8*f* and 1492*r* was performed to determine if the extracted DNA was pure enough for downstream applications and if the extracted RNA showed DNA contamination (Fig. 20).

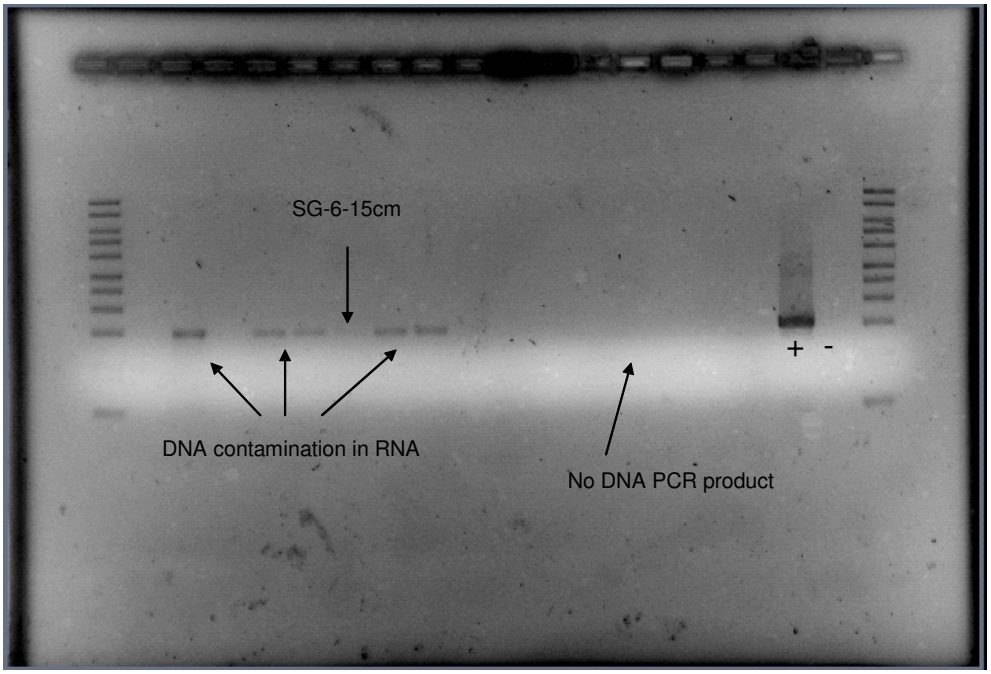

FIG. 20. 1% agarose gel image showing DNA contamination of RNA and DNA PCR product for SG-6.

The PCR reaction determined that several of the RNA samples were contaminated with carryover DNA and that the DNA samples were not pure enough to produce PCR product. The RNA samples were treated with RQ1 DNase (Promega Corp., Madison, Wis.) to degrade the DNA that was carried over during the RNA extraction. After the DNase digestion (30-40 minutes), a phenol-chloroform-isoamyl alcohol extraction

followed by an ethanol precipitation was performed as described in Sambrook et al (49). The RNA samples were stored at -80°C for later analysis.

The DNA samples were cleaned with the WIZARD DNA Cleanup System (Promega) according to the manufacturer's protocol. Regular PCR using the bacterial-specific primers 8*f* and 1492*r* (targeting the 16S rRNA gene) was performed on the DNA samples again (Fig. 21).

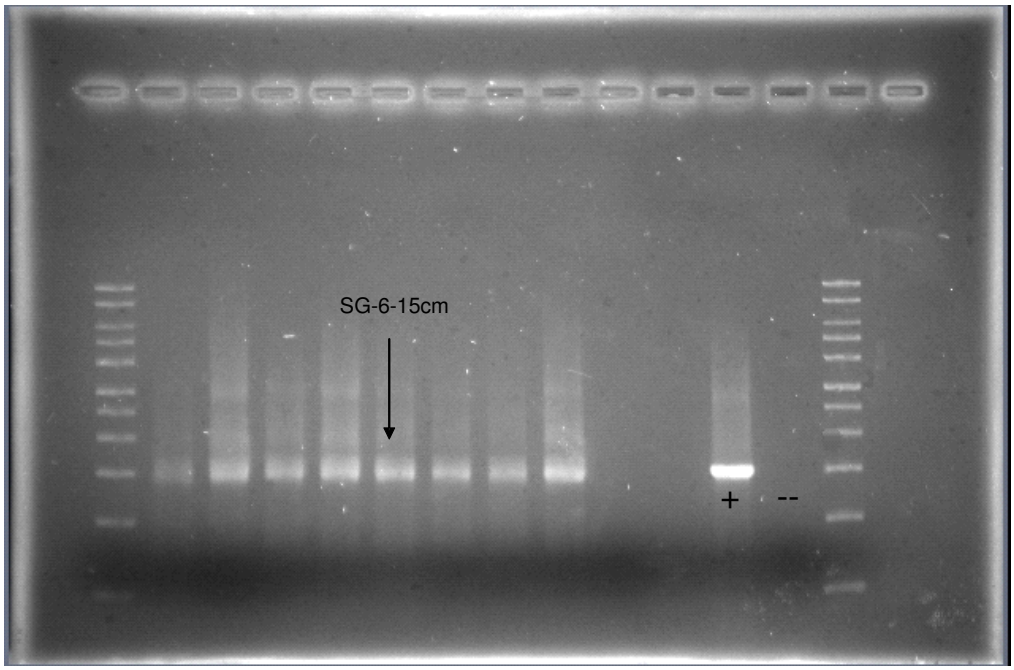

FIG. 21. 1% agarose gel image showing DNA PCR product for SG-6.

Reverse transcriptase PCR was performed on the RNA samples using the same bacterial-specific primers 8*f* and 1492*r*. RT-PCR was performed with the Access-Quick RT-PCR system (Promega) (Fig. 22). After RT-PCR analysis SG-6-15cm was chosen for the RNA/DNA clone libraries. Both PCR products (DNA and cDNA) were purified with the

WIZARD PCR Preps DNA Purification System (Promega) according to the manufacturer's protocol. Both the DNA and cDNA PCR products were aliquoted and stored at -80°C for later analysis.

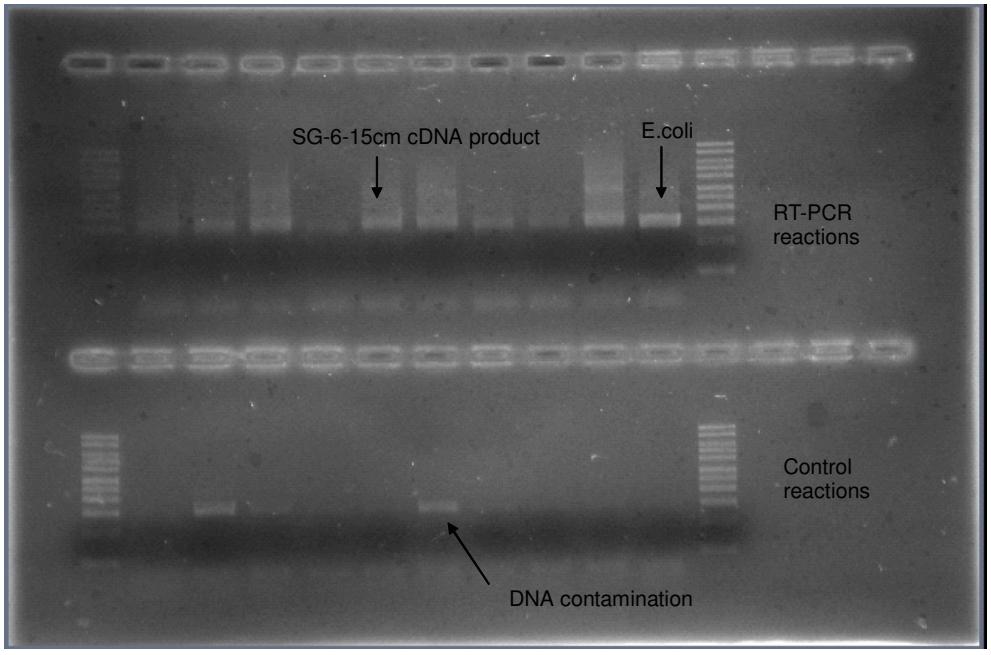

FIG. 22. 1% agarose gel image showing RT-PCR and control reactions for SG-6. Control reactions are missing the AMV Reverse Transcriptase enzyme and if positive indicate DNA contamination.

IV. 2. 3. 16S rRNA Gene Clone Library Construction with DNA and cDNA

16S rRNA gene clone libraries were constructed with the pGEM-T-Easy vector system (Promega). The pGEM-T-Easy vector system is a convenient system for the cloning of PCR products. The vector for this particular system has a 3´ terminal thymidine at both ends. These 3´-T overhangs at the insertion site (of the DNA fragment) greatly improve ligation efficiency by preventing recircularization of the vector (45). The Taq DNA polymerase (Roche) used in our PCR reactions added a single 3´ terminal adenosine to

the ends of the amplified fragments. The fact that both the vector and the DNA fragment have so called “sticky ends” enhances ligation efficiency as well. The pGEM-T-Easy vector also contains a α-peptide coding region. The α-peptide is inactivated when a DNA fragment is inserted into the vector and this inactivation allows recombinant clones to be identified by color screening on indicator plates (45).

The ligation reactions were set up as follows: 5µl 2x Rapid Ligation Buffer T4 DNA Ligase, 1µl pGEM-T-Easy Vector (50ng), 2µl cleaned PCR product (bright band on agarose gel) or 3µl cleaned PCR product (weak band on agarose gel), 1µl T4 DNA Ligase (3 Weiss units/µl), and PCR water to a final volume of 10µl. The reactions were mixed by pipetting and incubated overnight at 4°C for maximum number of transformants.

JM109 High Efficiency Competent Cells (Promega) were used for the transformations. It is essential to use competent cells with a transformation efficiency of at least 10^8 cfu/µg (cfu = colony forming units) in order to obtain a reasonable number of colonies. JM109 cells are guaranteed to have a transformation efficiency of at least 10^8 cfu/µg. Ligation reaction tubes were centrifuged to collect contents at the bottom. 2µl of each ligation reaction was added to a 15ml sterile Falcon tube on ice. JM109 High Efficiency Competent Cells (Promega) were removed from the -80°C freezer and thawed for approximately five minutes. 50µl of cells were carefully transferred into each 15ml sterile Falcon tube. 15ml tubes were gently petted to mix and placed on ice for 20

minutes. The cells were then heat-shocked for 50 seconds in a water bath at exactly 42°C. The tubes were immediately returned to ice for two minutes. 950µl room temperature SOC medium (100ml contains 2g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} stock (filter-sterilized), and 1ml 2M glucose (filter-sterilized)) was added to each tube. Tubes were incubated for 1.5 hours at 37°C with shaking (150rpm). 100µl of each transformation culture were plated onto duplicate or triplicate LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature). These particular agar plates allowed for color screening of the recombinant clones. The plates were incubated overnight at 37°C. The next day, the plates were color screened. White colonies contained the vector insert and blue colonies did not. The largest white colonies were transferred with sterile toothpicks onto fresh LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature) and incubated overnight at 37°C. The next day, half of each colony was transferred with a sterile toothpick into 0.5ml tubes containing 24µl "2 x cracking buffer" (50 ml contain 1ml 5M NaOH, 1ml 0.5M EDTA, 5ml 10% SDS, 5ml 100% Glycerol, and 38ml deionized water). The "cracking buffer" digested the competent cells, hence releasing the plasmid (vector containing DNA fragment). The entire 24µl were run on a 1% agarose gel at 100V, stained with ethidium bromide, and visualized under UV light. This step allowed us to check that the DNA fragment inserted into the vector was of correct size. The remaining halves of each colony were incubated again overnight at 37°C and transferred with a sterile toothpick onto fresh LB/ampicillin/IPTG/X-Gal plates (equilibrated to room temperature). The selected colonies were transferred with a sterile flaming loop to

15ml sterile Falcon tubes containing 5ml LB broth. The tubes were incubated overnight at 37°C with shaking (150rpm) until cloudy. The next day, the tubes were centrifuged at 4000 x g for two minutes at room temperature to collect the cell pellet. The supernatant was decanted and 1ml of 50mM EDTA (ethylenediaminetetraacetic acid) was added to wash the cell pellet. The cell pellet was resuspended by pipetting and centrifuged as before. The supernatant was decanted and the cell pellet was stored at -20°C until plasmid extraction. The plasmids were extracted using the E.Z.N.A. Plasmid Extraction Kit (Omega BioTek, Atlanta, GA). The plasmids were extracted according to the manufacturer's protocol. The extracted plasmids containing the desired DNA fragment were stored at -20°C for later analysis. PCR was performed to ensure that the extracted plasmids contained the 16S rDNA fragment and that they were pure enough for downstream analyses. The PCR reactions were 50µl in volume and contained 45µl PCR Supermix (Invitrogen, Carlsbad, CA), 0.5µl of primer 8*f* (10µmol), 0.5µl of primer 1492*r* (10µmol), 1µl BSA, 2µl PCR water, and 1µl plasmid DNA. PCR conditions were the same as previously described.

IV. 2. 4. ARDRA

Each individual clone was subjected to amplified ribosomal DNA restriction analysis (ARDRA) in order to characterize the 16S rRNA gene diversity within each clone library (32, 59). ARDRA reactions were as follows: 10µl PCR product (plasmid DNA), 0.75µl HaeIII restriction enzyme (7.5 units) (Promega), 0.75µl RsaI restriction enzyme (7.5 units) (Promega), 1.5µl 10x Buffer C (Promega), 0.15µl BSA (Promega), and

1.85µl PCR water for a final reaction volume of 15µl. ARDRA reactions were vortexed and centrifuged briefly and incubated 37°C for 4 hours. The resulting ARDRA patterns were separated on an 8% acrylamide gel [19:1, acrylamide / bis-acrylamide] using the BIORAD D-Code DGGE system (BioRAD, Hercules, CA). The pGEM DNA Marker (Promega) was used as the standard size ladder. The gels were run at 120V and 40°C for approximately 3 hours. The gels were stained with ethidium bromide and visualized under UV light. ARDRA patterns were analyzed using the GelCompar software program (Applied Maths, Inc., Austin, TX). The cluster analysis method used was the comparative numerical analysis with the unweighted pair group method using arithmetic averages (UPGMA). Based on this cluster analysis one or in some cases several representatives of each ARDRA pattern group from all clone libraries were selected for sequencing.

IV. 2. 5. Sequencing and Phylogenetic Analysis

Sequencing was performed at the DNA Analysis Facility on Science Hill at Yale University. Sequence data were first "blasted" in GenBank (http://www.ncbi.nlm.nih.gov) to identify the most similar sequences and then analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). This software is commonly used when analyzing 16S rRNA gene diversity. Dendrograms were reconstructed for the phylogenetic analysis. The frequencies of 16S rRNA gene phylotypes determined by ARDRA and subsequent sequencing (i.e., those sharing >97% identity) were used for analysis of diversity. Shannon's index for diversity (H') was

calculated according to the method of Zar (65). Shannon's index for diversity is by far the most commonly used diversity index. It takes into account the number of species but also the abundance of each species. Rarefaction curves were interpolated with the freeware program Analytic Rarefaction 1.3 (http://www.uga.edu/_strata/software/index.html). Rarefaction allows one to calculate species richness for a given number of sampled individuals. Rarefaction curves show the number of species as a function of the number of individuals sampled. Hence, a steep slope indicates that a fraction of the species diversity has not been sampled whereas a flattening slope indicates that diversity has been sampled well. Coverage of the clone libraries was estimated as described by Mullins et al. (39). Coverage was derived from the equation

$$C = 1 - (n_1/N)$$

where, n_1 is the number of clones that occurred only once and N is the total number of clones examined. This value is conservative, but excludes variation introduced by PCR artifacts and heterogeneities in rDNA gene families (39).

IV. 3. Results and Discussion

IV. 3. 1. rDNA versus rcDNA

Metabolically active and non-active members of the microbial community in the HSC sediments were examined by comparing clone libraries constructed from DNA (rDNA) and RNA (rcDNA) from the same sediment sample. We selected station SG-6 (Ch. I; Fig. 3) because it was located near a historic paper mill and Suarez et al (57) showed that

it had high dioxin concentrations. We selected a depth of 15cm since it was comparable to other depths used in this study (see Chapter III). Table 25 shows the bacterial diversity detected with both the 16S rDNA (DNA) and rcDNA (RNA) clone libraries.

TABLE 25. Bacterial diversity for station SG-6-15cm. Bacteria marked green were found in both the 16S rDNA (DNA) and rcDNA (RNA) clone libraries.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Alphaproteobacteria		
Uncultured alpha proteobacterium clone DPC255	X	
Betaproteobacteria		
uncultured bacterium clone MidBa16 (betaproteobacteria)	X	
uncultured bacterium clone MidBa40 (betaproteobacteria)	X	
uncultured bacterium clone BotBa80 (betaproteobacteria)	X	
Uncultured bacterium clone SS-73 (Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; Thiobacillus)	X	
Uncultured bacterium clone SS-88 (Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae)	X	
Uncultured bacterium clone KD5-121 (betaproteobacteria)	X	
Uncultured Hydrogenophilaceae bacterium clone D10_45 (Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae)	X	X
Uncultured bacterium clone Rap1_6C (Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae; Thiobacillus)	X	
Uncultured bacterium clone 1-5 (betaproteobacteria)	X	
Uncultured beta proteobacterium clone DR938CH110701SACH96	X	X
Uncultured bacterium clone SZB2 (betaproteobacteria)	X	
Uncultured bacterium clone BANW722 (betaproteobacteria)		X
Uncultured bacterium clone pfp37 (betaproteobacteria)		X
Uncultured beta proteobacterium clone 56S_1B_48		X
Uncultured beta proteobacterium clone 56S_1B_81		X
Uncultured bacterium clone DR-10 (betaproteobacteria)		X
uncultured bacterium clone TopBa9 (betaproteobacteria)		X
Uncultured bacterium clone FAC45 (betaproteobacteria)		X
Uncultured beta proteobacterium clone A2-4c11		X
Gammaproteobacteria		
Uncultured gamma proteobacterium clone Belgica2005/10-140-8	X	
Uncultured gamma proteobacterium clone Belgica2005/10-140-20		X
uncultured bacterium clone MidBa19 (gammaproteobacteria)	X	
Uncultured bacterium clone 35-8 (Gammaproteobacteria; Thiotrichales; Thiotrichaceae)	X	

TABLE 25. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Uncultured bacterium clone SZB7 (Gammaproteobacteria; Chromatiales)		X
Uncultured bacterium clone SZB11 (gammaproteobacteria)	X	
Uncultured bacterium clone SZB16 (gammaproteobacteria)	X	X
Uncultured bacterium clone SZB30 (Gammaproteobacteria; Chromatiales)		X
uncultured bacterium clone TopBa15 (gammaproteobacteria)	X	
Uncultured gamma proteobacterium clone MSB-4D7	X	
Uncultured gamma proteobacterium clone MSB-4G2		X
Coxiella burnetii RSA 331 (Gammaproteobacteria; Legionellales; Coxiellaceae; Coxiella)	X	
C.orbicularis symbiont (gammaproteobacteria)		X
Uncultured bacterium clone JH-WHS168 (Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae)		X
Uncultured gamma proteobacterium clone Sylt 39		X
Uncultured bacterium clone DB-47 (gammaproteobacteria)		X
Uncultured gamma proteobacterium clone CA08		X
Uncultured gamma proteobacterium clone: pItb-vmat-3		X
Uncultured gamma proteobacterium clone 3G02-10		X
Agricultural soil bacterium clone SC-I-73 (gammaproteobacteria)		X
Deltaproteobacteria		
Uncultured bacterium clone 11bavF12 (deltaproteobacteria)	X	
Uncultured bacterium clone MD2902-B140 (deltaproteobacteria)	X	
Uncultured bacterium clone MD2896-B233 (deltaproteobacteria??)	X	
Uncultured bacterium clone MD2896-B143 (deltaproteobacteria???)	X	
Uncultured bacterium clone MSB-2B1 (deltaproteobacteria)		X
Uncultured bacterium clone MSB-2C9 (deltaproteobacteria)		X
Uncultured bacterium clone MSB-2E2 (deltaproteobacteria)		X
Uncultured delta proteobacterium clone MSB-4H8		X
Uncultured delta proteobacterium clone MSB-5A4	X	X
Uncultured bacterium clone MSB-5A5 (deltaproteobacteria)		X
Uncultured delta proteobacterium clone MSB-5C4	X	X
Uncultured delta proteobacterium clone MSB-5C5		X
Uncultured delta proteobacterium clone MSB-5D12	X	X
Uncultured delta proteobacterium clone MSB-5D8		X
Uncultured delta proteobacterium clone MSB-5bx5	X	
Uncultured delta proteobacterium clone XME70	X	X
Uncultured bacterium clone AN07BC1_15cmbsf_105B (deltaproteobacteria)	X	
Uncultured delta proteobacterium clone Hyd89-29	X	
Uncultured bacterium clone 30f10 (deltaproteobacteria)	X	
Uncultured bacterium clone Amb_16S_1529 (deltaproteobacteria)	X	
Uncultured hydrocarbon seep bacterium GCA017 (deltaproteobacteria)	X	
Uncultured delta proteobacterium clone CB1129	X	

TABLE 25. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Uncultured bacterium clone 31c10 (Deltaproteobacteria; Syntrophobacterales)	X	
Uncultured organism clone MAT-CR-M1-A01 (deltaproteobacteria)	X	
Uncultured bacterium ODP1230B1.06 (deltaproteobacteria)	X	
Uncultured bacterium clone: AMG24B-12 (probably proteobacteria)	X	
Uncultured bacterium clone Nubeena16 (deltaproteobacteria)		X
Uncultured delta proteobacterium clone Nubeena225		X
Uncultured Syntrophaceae bacterium clone D15_21 (Deltaproteobacteria; Syntrophobacterales; Syntrophaceae)		X
Uncultured bacterium clone TTA_H101 (Deltaproteobacteria; Syntrophobacterales; Syntrophaceae; Syntrophus)		X
Uncultured bacterium clone 8bav_A9 (deltaproteobacteria)		X
Uncultured bacterium clone zEL26 (deltaproteobacteria)		X
Uncultured delta proteobacterium clone Hyd89-22		X
Uncultured delta proteobacterium clone Belgica2005/10-130-31 (Deltaproteobacteria; Desulfobacterales)		X
uncultured bacterium clone BotBa59 (Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae)		X
Uncultured Desulfobacteraceae bacterium clone cLaKi-JM47 (Deltaproteobacteria; Desulfobacterales; Desulfobacteraceae)		X
Uncultured bacterium clone 10BAV_C9_ready (deltaproteobacteria)		X
Uncultured bacterium clone 655066 (deltaproteobacteria?)		X
Uncultured delta proteobacterium clone SI29		X
Uncultured bacterium clone c5LKS37 (deltaproteobacteria)		X
Uncultured Geobacter sp. clone VHS-B3-70 (Deltaproteobacteria; Desulfuromonadales; Geobacteraceae; Geobacter)		X
Uncultured delta proteobacterium clone SL13		X
Uncultured delta proteobacterium clone: HMMVPog-13		X
Uncultured delta proteobacterium clone: HMMVPog-19		X
Uncultured bacterium clone: Baqar.Sed.Eubac.8. (deltaproteobacteria)		X
Uncultured soil bacterium clone HS9-74 (deltaproteobacteria)		X
Uncultured delta proteobacterium MERTZ_2CM_130		X
Uncultured delta proteobacterium clone 88FS		X
Olavius algarvensis sulfate-reducing endosymbiont (deltaproteobacteria)		X
Agricultural soil bacterium clone SC-I-55 (deltaproteobacteria)		X
Uncultured bacterium clone Hast2_4 (deltaproteobacteria)		X
Uncultured bacterium clone JG2 (proteobacteria?, maybe delta)	X	
Proteobacteria		
Uncultured bacterium clone S-Btb7_40 (proteobacteria)		X
Uncultured bacterium clone: #5-4 (proteobacteria)		X
Uncultured bacterium clone WM91 (probably proteobacteria)		X

TABLE 25. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Acidobacteria		
Uncultured Acidobacteria bacterium isolate OTU14/APA	X	
Uncultured bacterium clone 12A-10 (Acidobacteria)	X	
Uncultured bacterium clone Fitz2_17 (Acidobacteria)	X	
Uncultured Acidobacteria bacterium clone MSB-3G5		X
Uncultured bacterium clone CI75cm.2.17 (Acidobacteria)		X
Chloroflexi		
Uncultured Chloroflexi bacterium clone HCM3MC91_6G_FL	X	
Uncultured green non-sulfur bacterium clone P. palm C 37 (Chloroflexi)	X	
Uncultured Chloroflexi bacterium clone MSB-5bx1	X	X
Uncultured bacterium clone CI75cm.2.02 (Chloroflexi)	X	
Uncultured Chloroflexi bacterium clone Belgica2005/10-130-20	X	
Uncultured eubacterium t0.6.f (Chloroflexi, Dehalococcoides)	X	
Uncultured bacterium clone SLB319 (Chloroflexi)	X	
Uncultured organism clone MAT-CR-P1-H07 (Chloroflexi)	X	
Uncultured organism clone MAT-CR-P5-G09 (Chloroflexi)		X
Uncultured bacterium clone TfP20L72 (Chloroflexi?)	X	
Uncultured bacterium clone GIF9 (Chloroflexi)	X	
Uncultured bacterium clone FS117-62B-02 (Chloroflexi)	X	
uncultured bacterium clone BotBa28 (Chloroflexi, Dehalococcoides)		X
Uncultured green non-sulfur bacterium clone MBAE68 (Chloroflexi)		X
Uncultured bacterium clone:RB345 (Chloroflexi)		X
Spirochetes		
Uncultured bacterium clone S11-88 (spirochete)	X	
Uncultured bacterium clone MD2896-B83 (spirochete)	X	
Uncultured bacterium clone c5LKS77 (spirochete)	X	
uncultured bacterium SJA-88 (spirochete)	X	
Uncultured spirochete clone LH042	X	
Uncultured bacterium clone: HSM-SS-024 (spirochete)		X
Uncultured Spirochaetales bacterium clone COREB32		X
Nitrospirae		
Uncultured Nitrospirae bacterium clone MSB-4D12	X	
Uncultured Nitrospirae bacterium clone MSB-3B5	X	
Uncultured bacterium clone FW114 (Nitrospirae)	X	
Uncultured bacterium clone 35-52 (Nitrospirae)	X	
Uncultured bacterium clone TP98 (Nitrospirae)	X	
Uncultured bacterium clone JG37 (Nitrospirae)	X	

TABLE 25. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Actinobacteria		
Uncultured bacterium clone 35-13 (actinobacteria)	X	
Uncultured bacterium clone ODP1230B3.20 (Actinobacteria)	X	
Uncultured actinobacterium clone D15_07	X	
Uncultured actinobacterium clone: Y194	X	
Uncultured actinobacterium clone MSB-5D2		X
Uncultured bacterium clone: #2-3 (actinobacteria?)		X
Firmicutes		
Uncultured bacterium clone FFCH17943 (Firmicutes?)	X	
Thermoanaerobacter tengcongensis (Firmicutes; Clostridia; Thermoanaerobacteriales; Thermoanaerobacteriaceae; Thermoanaerobacter)	X	
Uncultured bacterium clone MD2905-B17 (Firmicutes?)	X	
Uncultured bacterium clone Hyd24-32 (Firmicutes)		X
Chlorobi		
Uncultured bacterium clone 8bav_H5_arb (Chlorobi?)	X	
Uncultured Chlorobi bacterium clone: 397	X	
Uncultured Bacteroidetes/Chlorobi group bacterium clone 3B1820-44	X	
Uncultured bacterium clone PS-Ba73 (Bacteroidetes)	X	
Planctomycetes		
Uncultured bacterium clone 60_st5_10-12cm (planctomycete)	X	
Uncultured bacterium clone PMMV-Bac19 (planctomycete)	X	
Verrucomicrobia		
Uncultured Verrucomicrobia bacterium clone LD1-PB1	X	
Uncultured Verrucomicrobia bacterium clone LD1-PB12		X
Uncultured bacterium clone lka36 (Verrucomicrobia)	X	
Uncultured Verrucomicrobia bacterium clone Dover171		X
Candidate Division OP8		
Uncultured bacterium clone 68_st3_10-12cm (Candidate Division OP8)	X	
Uncultured candidate division OP8 bacterium clone HS9-30	X	
Uncultured bacterium clone pLW-103 (Candidate Division OP8)	X	
Uncultured bacterium clone A1 (Candidate Division OP8)		X
Candidate Division OP3		
Uncultured bacterium clone GIF19 (candidate division OP3)	X	
Uncultured bacterium clone MD2894-B10 (Candidate Division OP3)	X	

TABLE 25. Continued.

Closest 16S rRNA relative identified in GenBank and ARB database	rDNA	rcDNA
Candidate Division OP10		
Uncultured bacterium clone: AMG24B-02 (candidate division OP10)	X	
Candidate Division TG3		
Uncultured candidate division TG3 bacterium	X	
Candidate Division AC1		
Uncultured bacterium clone MD2896-B64 (Candidate Division AC1??)	X	
Candidate Division WS3		
Uncultured bacterium clone Chun-s-19 (Candidate Division WS3)		X
Uncultured bacterium clone SHA-71 (Candidate Division WS3)		X
Cytophaga		
uncultured bacterium clone MidBa45 (Cytophaga)		X
Uncultured Cytophaga sp. clone VHS-B5-77		X
Cyanobacteria		
Cyanobium sp. JJ2-3 (Cyanobacteria; Chroococcales; Cyanobium)		X
Aggregate-forming unicellular cyanobacterium LLi5		X
Fusobacteria		
Unidentified bacterium clone: NKB19 (Fusobacteria)		X
Uncultured bacterium clone MD2896-B272 (Fusobacteria)		X
Unknown		
Uncultured bacterium clone 5bav_B3arb (unknown)	X	
Uncultured bacterium clone MSB-2F12 (unknown)	X	
Uncultured bacterium clone MSB-4E2 (unknown)	X	
Uncultured bacterium clone SLB616 (unknown)	X	
Uncultured bacterium clone 23g04 (unknown)		X
Uncultured bacterium clone SRRT67 (unknown)		X
Unidentified bacterium clone TK-SH22 (unknown)		X
Uncultured forest soil bacterium clone DUNssu145 (unknown)		X
Uncultured organism clone MAT-CR-H5-C03 (unknown)		X

IV. 3. 2. Phylogenetic Analysis of the Clone Libraries

Phylogenetic Analysis of the Clone Library Derived from DNA

For the DNA-derived (rDNA) clone library 111 clones were examined. The majority of the cloned sequence types derived from DNA templates clustered within the *Proteobacteria* (50%). *Deltaproteobacteria* was the most frequently detected lineage (24.3%), followed by *Betaproteobacteria* (17.2%), *Gammaproteobacteria* (7.2%), and *alphaproteobacteria* (0.9%). The closest relatives to almost all of the proteobacteria clones could only be identified down to the subdivision level (i.e. delta, beta, etc.); however, four of the clones that clustered within the *Betaproteobacteria* also clustered within the genus *Thiobacillus*. Members of the genus *Thiobacillus* are sulfur-oxidizing chemolithotrophs (Madigan et al, 2003). The next biggest phylogenetic groups are the *Chloroflexi* (10.8%) and the *Nitrospirae* (7.2%). A relatively small number of clones clustered with the phyla *Spirochetes* (4.5%), *Actinobacteria* (3.6%), *Chlorobi* (3.6%), *Holophaga/Acidobacteria* (2.7%), *Firmicutes* (2.7%), *Planctomycetes* (1.8%), and the *Verrucomicrobia* (1.8%). 8.1% of the cloned sequence types clustered with five candidate divisions, namely OP8 (2.7%), OP3 (1.8%), OP10 (1.8%), TG3 (0.9%), and AC1 (0.9%). 3.6% of the clones did not have a known closest relative. Overall, the cloned sequence types clustered with 18 known phylogenetic groups.

Phylogenetic Analysis of the Clone Library Derived from RNA

For the RNA-derived (rcDNA) clone library 104 clones were examined. Almost two thirds of the cloned sequence types derived from RNA templates clustered within the

Proteobacteria (72%). *Deltaproteobacteria* was by far the most frequently detected lineage (41.3%), followed by *Gammaproteobacteria* (17.3%), *Betaproteobacteria* (11.5%). 1.9% of the clones only clustered at the phylum level of *Proteobacteria*. *Alphaproteobacteria* was absent in this clone library. The closest relatives to almost all of the proteobacteria clones could only be identified down to the subdivision level (i.e. delta, beta, etc.); however, three of the clones that clustered within the *Deltaproteobacteria* also clustered within the family *Desulfobacteraceae* (sulfate-reducing bacteria). One clone clustered within the genus *Geobacter*, which is known oxidize organic compounds, metals, and petroleum products. Two clones clustered within the family *Syntrophaceae* (sulfate-reducing bacteria, includes the genus *Desulfomonile*). The next biggest group in the clone library clustered with the *Chloroflexi*, which includes *Dehalococcoides* (4.8%). The remaining clones clustered with the phyla *Spirochetes* (1.9%), *Actinobacteria* (1.9%), *Holophaga/ Acidobacteria* (1.9%), *Verrucomicrobia* (1.9%), *Cytophaga* (1.9%), *Fusobacteria* (1.9%), *Cyanobacteria* (1.9%), and *Firmicutes* (1.0%). 2.9% of the clones clustered with candidate divisions, namely OP8 (1.0%) and WS3 (1.9%). 5.9% of the clones did not have a known closest relative. Overall, the cloned sequence types clustered with 14 known phylogenetic groups.

Delta-, *Beta-*, and *Gammaproteobacteria* were found in the DNA-derived as (rDNA) well as the RNA-derived (rcDNA) clone library. There were almost twice as many *Deltaproteobacteria* in the rcDNA clone library compared to the rDNA one. It is not

surprising that *Deltaproteobacteria* are abundant in HSC sediments, but the fact that they almost doubled in abundance in the rcDNA clone library, which indicates that they are active, is quite significant. In both clone libraries *Chloroflexi* was the next biggest group after the *Proteobacteria*. In the rDNA clone library, Chloroflexi comprised 10.8%, whereas in the rcDNA clone library it comprised about half that (4.8%). This indicates that roughly half of the rDNA-detected Chloroflexi might not be active members of the microbial community in the HSC sediments. Several phyla occurred in both clone libraries, namely *Spirochetes*, *Holophaga/Acidobacteria*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia*, *Nitrospirae*, *Planctomycetes*, and *Chlorobi* were only found in the rDNA clone library. *Cytophaga*, *Fusobacteria*, and *Cyanobacteria* were only found in the rcDNA clone library. Five candidate divisions were found in the rDNA clone library, but only one (OP8) also occurred in the rcDNA clone library. On the other hand one candidate division (WS3) was only found in the rcDNA clone library. Overall, eight clones were found in both clone libraries. Four of these clustered with the *Deltaproteobacteria*, two with the *Betaproteobacteria*, one clustered with the *Gammaproteobacteria*, and one with the *Chloroflexi,* which includes *Dehalococcoides*. Almost all of the clones from both clone libraries were most similar to other uncultured clones. The fact that a few Chloroflexi clones were found in the rcDNA clone library is very promising since it indicates that members of the group *Chloroflexi/ Dehalococcoides* are active within the microbial community.

IV. 3. 3. Coverage, Diversity, and Rarefaction Analysis

For the DNA-derived (rDNA) clone library, coverage was 27.2% and 21.1% for the RNA-derived (rcDNA) clone library. Thus the data presented here would account for 27% (rDNA) and 21% (rcDNA) of the clones in a similar clone library of infinite size (39). Unfortunately, the available information does not permit us to estimate the diversity of the remaining 73% (rDNA) and 79% (rcDNA) of 16S rRNAs that are unaccounted for. This coverage is considerably lower and more like the coverage of the total bacterial diversity clone libraries discussed in Chapter III. This leads to the conclusion that in order to sufficiently cover diversity, well over 100 clones need to be examined. The clone libraries presented here represent a good starting point for further microbial diversity analyses of the metabolically active fraction present in HSC sediments. According to the Shannon's index for diversity (H'), total bacterial diversity for the rDNA clone library was 1.92 and 1.96 for the rcDNA clone library. Since the coverage calculations showed that only a small portion of the diversity was analyzed for the HSC clone libraries, it is expected that diversity will increase with more clones examined. Evenness, J', was 0.97 for the rDNA clone library and 0.98 for the rcDNA one. This indicates that total bacterial diversity is fairly evenly distributed. These numbers are also close to the diversity values calculated for the other total bacterial diversity clone libraries discussed in this thesis.

Rarefaction curves were also calculated (Figs. 23 and 24) and show that overall bacterial diversity has not been sampled well.

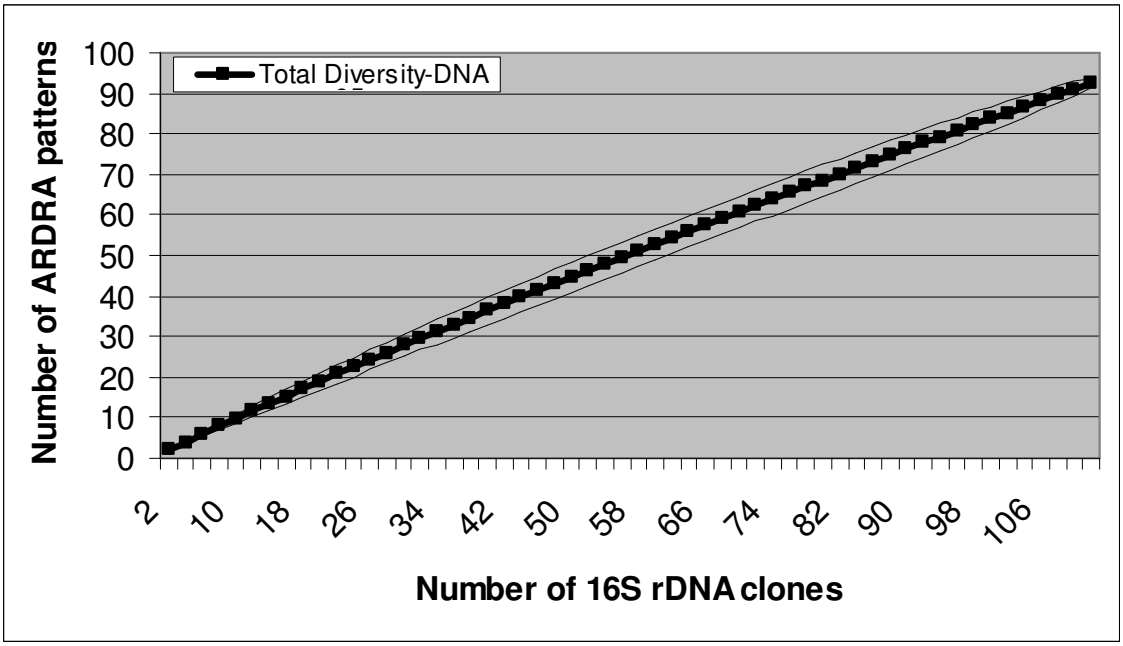

FIG 23. Rarefaction curve for the different ARDRA patterns of 16S rDNA clones for SG-6-15cm-DNA.

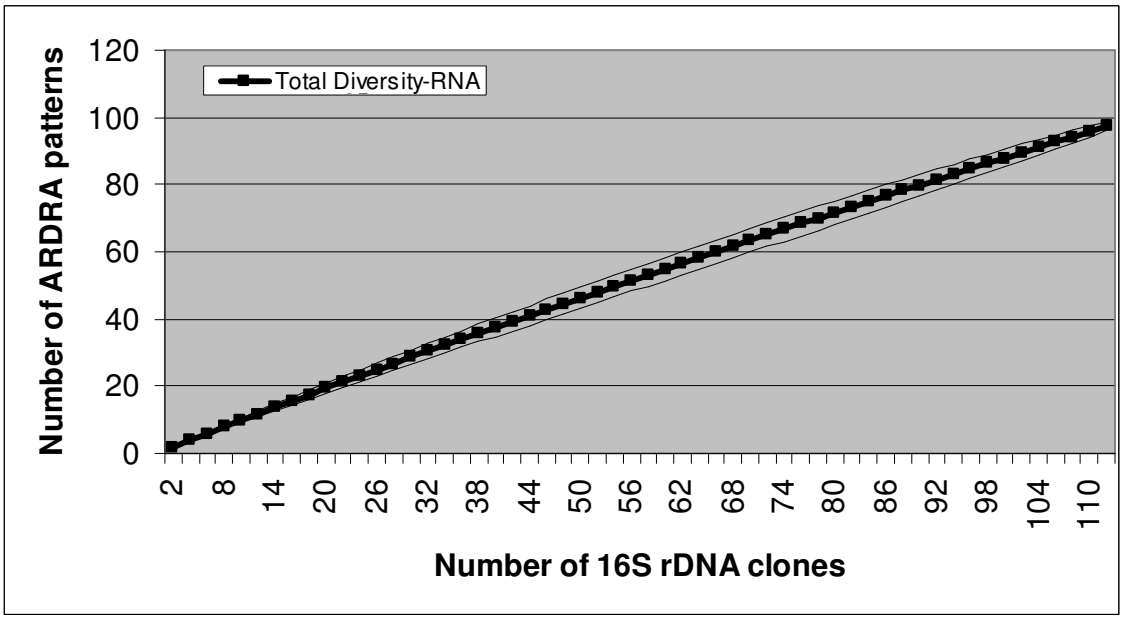

FIG 24. Rarefaction curve for the different ARDRA patterns of 16S rDNA clones for SG-6-15cm-RNA.

Since diversity was not sampled well when looking at individual clones, I investigated whether or not diversity is better sampled when considering phylogenetic groups. I calculated coverage, diversity, evenness, and a rarefaction curve considering 23 phylogenetic groups for the rDNA clone library and 20 phylogenetic groups for the rcDNA clone library. Coverage for the groups was 69.6% for rDNA and 70% for rcDNA clone libraries. This is a dramatic increase compared to 27% (rDNA) and 21% (rcDNA) for individual clones. This indicates that the phylogenetic groups are well covered within the sediment, but not their individual members. According the Shannon's index for diversity (H'), total bacterial diversity was 1.12 (rDNA) and 0.92 (rcDNA). This is significantly lower than 1.92 (rDNA) and 1.96 (rcDNA) for individual clones. Evenness (J') was 0.82 (rDNA) and 0.70 (rcDNA). These values are also lower compared to 0.97 (rDNA) and 0.98 (rcDNA) for individual clones. This evenness may actually represent the diversity a little better, since phylogenetic analysis showed that deltaproteobacteria was the dominant group. The rarefaction curves are also flattening, indicating that when considering phylogenetic groups, diversity is fairly well sampled by these clone libraries (Figs. 25 and 26).

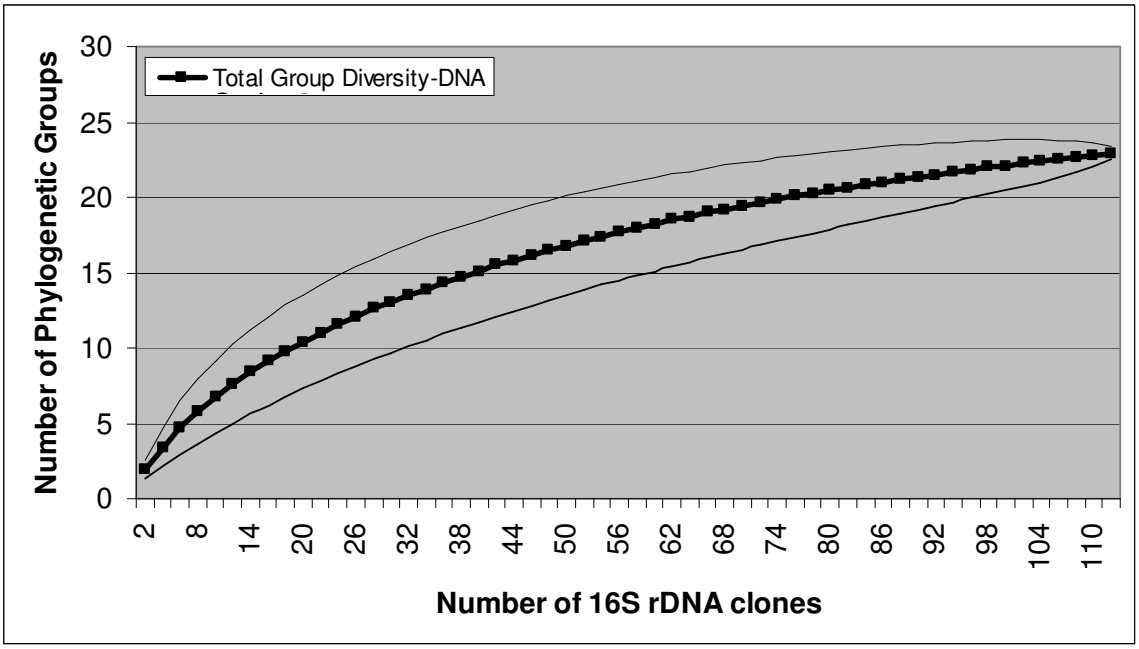

FIG 25. Rarefaction curve for the different phylogenetic groups of the 16S rDNA clones for the SG-6-15cm-DNA.

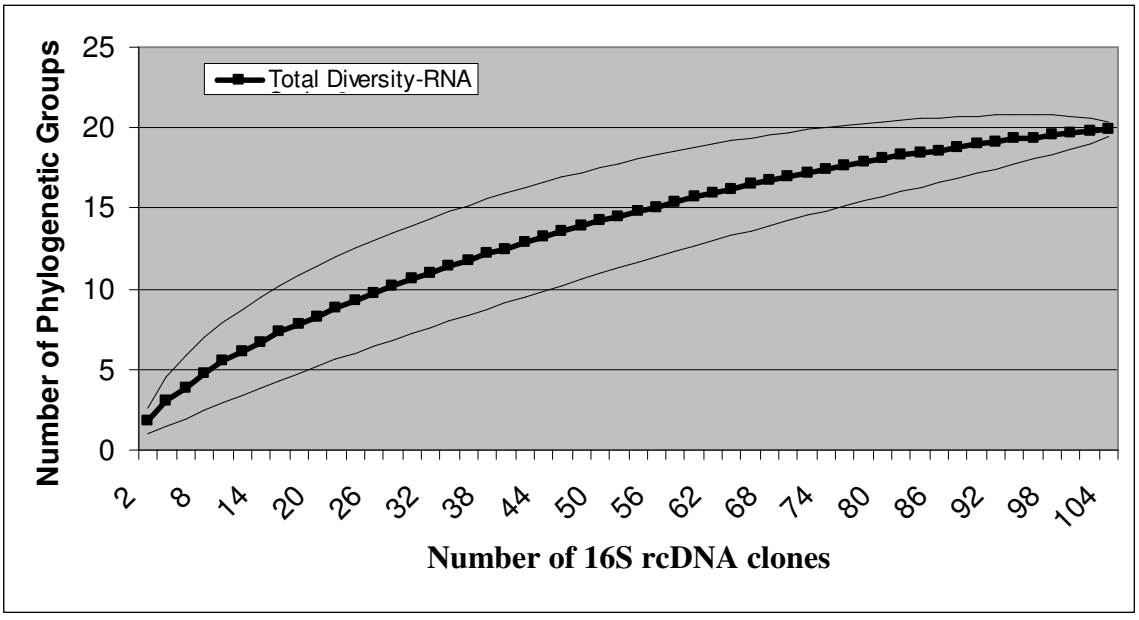

FIG 26. Rarefaction curve for the different phylogenetic groups of the 16S rcDNA clones for the SG-6-15cm-RNA.

IV. 3. 4. Conclusions

Total bacterial diversity within the sediments of station SG-6 along the HSC was very high. These results continue the trend of high bacterial diversity within the HSC sediments, as seen by the previous clone libraries (Chapter III). As indicated by the low coverage values of 27 and 21% for the rDNA and rcDNA clone libraries, respectively, neither clone library sampled bacterial diversity well. Thus we cannot validly conclude that the rcDNA and rDNA clone libraries obtained are truly representative of the active and total bacterial populations within HSC sediments. Problems with PCR amplification may have included inadequate primer specificity as well as inhibition by heavy metals, petroleum, humics and other chemicals in the sediments. In fact, poor sampling of species diversity in bacterial communities using molecular methods is not uncommon. For example, Miskin et al. (36) found only 26 and 5% coverage among RNA- and DNA-derived clones, respectively, from anoxic sediments from a productive freshwater lake in England due to undetermined factors.

Even though only a small fraction of the overall diversity of individual species is represented in these two clone libraries, the major phylogenetic groups (*Proteobacteria* and *Chloroflexi*) are represented well in both. Furthermore, these two major phylogenetic groups are also found in other clone libraries from the HSC sediments (see Chapter III). *Proteobacteria* and *Chloroflexi* make up the majority in both clone libraries, but to varying degrees. In the DNA-derived clone library, *Proteobacteria* account for 50% of the overall diversity, whereas in the RNA-derived clone library,

Proteobacteria account for 72%. This is a 31% increase and indicates that *Proteobacteria*, especially *Deltaproteobacteria,* are dominant in the metabolically active fraction of the bacterial population in HSC sediments. In contrast, *Chloroflexi* account for 11% of the diversity in the DNA-derived clone library and 5% in the RNA-derived one. This is a 55% decrease and indicates that even though *Chloroflexi* are the second most abundant bacterial group in HSC sediments; their metabolically active population is half the size of their total population. This could be due to several factors, including localized redox conditions, the biological availability of carbon sources (i.e. dioxins), the presence/absence of dechlorination stimulators (i.e. other halogenated compounds) (4), and competition with other dechlorinators (such as *Deltaproteobacteria*).

The remaining phylogenetic groups such as *Holophaga/Acidobacteria*, *Spirochetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, and various Candidate Divisions follow a pattern similar to that of *Chloroflexi*. These groups combined make up roughly 40 % of the overall diversity in the DNA-derived clone library, but only 23% in the RNA-derived one. This is a 42% decrease and indicates that the metabolically active populations of these groups are significantly smaller than their total populations. Overall, this shows how the metabolically active fraction in HSC sediments is dominated by the *Deltaproteobacteria* (41%). Just like *Chloroflexi*, members of the *Deltaproteobacteria* are also capable of dechlorination (for example, *Desulfomonile* and *Desulfuromonas*) as well as sulfate reduction (22, 33). Again, it is noteworthy that almost all the phylotypes from both clone libraries are most closely related to other uncultured phylotypes from

(contaminated) sediments worldwide. This phenomenon is often reported for 16S rRNA gene sequences recovered from environmental samples (36).

The observation that some rcDNA (RNA) phylotypes were not detected in the rDNA (DNA) clone library may be explained by the low coverage of the rDNA clone library. Since the rDNA clone library represents the total bacterial population and the rcDNA clone library represents the metabolically active bacterial population, one would expect every phylotype in the rcDNA clone library to be present in the rDNA clone library as well. Coverage for the rDNA and rcDNA clone libraries was 27 and 21%, respectively. These coverages are rather low and indicate poor sampling of species diversity. Larger clone libraries are needed to sample bacterial diversity well and validly conclude that the rcDNA and rDNA clone libraries obtained are truly representative of the active and total bacterial populations within HSC sediments.

This is the first study to report the composition of the metabolically active members of the bacterial community in HSC sediments. Even though phylogenetic surveys derived from DNA are informative, they cannot determine the ecological significance of the organisms from which the gene sequences were recovered, since DNA is known to persist in dead cells and extracellularly (36). RNA, on the other hand, is highly labile and rRNA levels have been correlated with cellular activity (35). Sequences recovered from an RNA template using RT-PCR imply that the source organisms were active *in situ* at the time of sampling or close to it (31, 36, 41). This is in contrast to DNA

templates where such a distinction cannot be made (35). Using rRNA as a means to study the metabolically active members of the bacterial community has been proposed for several years (Pichard and Paul, 1993) and is especially informative for bioremediation studies. Unfortunately, RNA is more difficult to isolate than DNA. This is due mainly to the rapid degradation of RNA by the enzyme RNase, which is both stable and ubiquitous (36). To date, most methods developed for RNA extraction from sediments have been rather lengthy and some also require expensive equipment (e.g. an ultracentrifuge). Miskin et al (36) developed a rapid method for the extraction of RNA from sediments; however, the developed method, which used sterile glass beads (0.17-0.18 mm diameter), only yielded reproducible rRNA fragments of about 530 base pairs. Such short fragments make phylogenetic analysis difficult. The RNA extraction method used in this study yielded rRNA fragments of about 1,100 base pairs, making phylogenetic analysis easier and more reliable.

Although the bacterial composition determined by this RT-PCR approach is not quantitative, it does suggest that the organisms represented by these sequences play an important metabolic role in the sediments (41). Hence, these results are promising with respect to *in situ* bioremediation in HSC sediments. This study confirms that members of the clade *Chloroflexi*, thought to include *Dehalococcoides*, are indeed part of the metabolically active fraction of the bacterial community in HSC sediments. Since these organisms are already present and active, it would make *in situ* bioremediation more feasible and successful. It would be interesting to construct and analyze a RNA-derived

clone library targeting the bacterial group *Dehalococcoides*. Such a clone library would shed more light on the active members of the bacterial community who are able to dechlorinate halogenated compounds, such as dioxin. Another possibility would be to specifically target dehalogenase genes. These genes have been shown to be responsible for the dechlorination of halogenated aromatic compounds (23, 53). Unfortunately, the dehalogenase gene responsible for the breakdown of dioxin has yet to be identified. Knowing, in more detail, which *Dehalococcoides* phylotypes are the most active within the HSC sediments will be helpful in determining which strains to focus on for *in situ* bioremediation.

CHAPTER V

CONCLUSIONS

V. 1. Conclusions

V. 1. 1. Presence of *Dehalococcoides* in HSC Sediments

Dehalococcoides was detected in every HSC sediment core, except the wetlands control site (FW1A) and the HSC cores that had PCR inhibition throughout. The sediment cores collected were anoxic within the first centimeter and all of them had a distinct rotten egg/petroleum/ chemical smell to them-to varying degrees. Since *Dehalococcoides* is strictly, anaerobic, one would only expect it in anaerobic environments. Table 26 summarizes the depths at which *Dehalococcoides* was first detected for each sediment core.

TABLE 26. Stations and the depth (in cm) at which *Dehalococcoides* was first detected.

Station	Sediment Depth (in cm) at which *Dehalococcoides* was first detected
11193	6
11270	3
15244	8
11261	30
13337	11
FW1A	Not detected
SG 1	Inhibition
SG 3	5
SG 4	2
SG 6	1
SG 7	3
SG 8	4

Detection of *Dehalococcoides* varied from 1cm (SG-6) to 30cm (11261). SG-6 1 cm could be a false positive, since primer set 1 did have some nonspecific amplification; however, sedimentary analysis revealed that the sediments of SG-6 had been mixed recently, increasing the likelihood of the detection of *Dehalococcoides* in the upper sediment layers. *Dehalococcoides* was detected further down in the sediment core at depths 6cm through 10cm. Since the SG-6 sediment core was taken from the so called 'Dioxin Pit', it is very likely that *Dehalococcoides* is present already at 1 cm.

There seems to be a minimum dioxin concentration of about 3 total TEQ ng/kg dry weight needed for *Dehalococcoides* to occur. This is a low concentration compared to dioxin concentrations in the HSC sediments (station 11270: ~ 30 total TEQ ng/kg dry weight; stations 11193, 13337, SG 3, and SG 4 ~ 8 total TEQ ng/kg dry weight) *Dehalococcoides* was not detected in the wetlands control site (FW1A), where dioxin concentrations were below 1 total TEQ ng/kg dry weight. Table 27 shows the depths and (estimated) dioxin concentrations where *Dehalococcoides* was first detected.

TABLE 27. Stations, depths (in cm), and dioxin concentrations at which *Dehalococcoides* were first detected.

Station	**Sediment Depth (in cm) at which *Dehalococcoides* was first detected**	**Estimated Age of Sediment (yr)**	**Dioxin concentration (total TEQ ng/kg dry wt) at which *Dehalococcoides* was first detected**
11193	6	~ 2	~ 8
11270	3	3.48	~ 30
15244	8	7.12	~ 4-5
11261	30	5.2	2.98
13337	11	6.3	8.22
FW1A	Not detected	-	below 1
SG 1	Inhibition	-	-
SG 3	5	Data not yet available	~ 8-10
SG 4	2	Data not yet available	~ 7-8
SG 6	1	Data not yet available	Data not yet available
SG 7	3	Data not yet available	Data not yet available
SG 8	4	Data not yet available	Data not yet available

Dioxin concentrations varied widely and it seemed that there was only a minimum concentration of below 1 total TEQ ng/kg dry weight where *Dehalococcoides* was not detected. Since the detection of *Dehalococcoides* was based on molecular methods utilizing DNA-derived templates, one cannot validly conclude that the organisms were active and/or alive at all the depths at which they were detected, including the ones with very low dioxin concentrations. However, the since *Dehalococcoides* was extensively detected throughout all of the stations analyzed along the HSC makes it very likely that members of the group were indeed active in at least deeper parts of the sediment cores, i.e. the ones which exhibited higher dioxin concentrations. Bunge et al (14) showed that *Dehalococcoides* sp. strain CBDB1 is capable of reductively dechlorinating 1,2,3,4-TCDD and 1,2,3,7,8-PeCDD. Fennell et al (15) showed that *Dehalococcoides ethenogenes* strain 195 is also able to dechlorinate 1,2,3,4-TCDD but is unable to

dechlorinate 2,3,7,8-TCDD. Neither study investigated whether or not *Dehalococcoides* require a minimum or maximum concentration of substrate in order to thrive and grow. To my knowledge, no studies have been conducted to determine whether or not there is a concentration at which dioxins and other halogenated compounds become toxic to these bacteria. Since *Dehalococcoides* use dioxins and halogenated compounds as an energy source, this is unlikely (2).

There also seems to be no correlation with depth, since detection depths ranged from 2-30cm, rather the occurrence of *Dehalococcoides* seems to depend on as of yet unidentified factors. Sedimentary and biogeochemical data were not available for all cores, making it difficult to link the presence of *Dehalococcoides* to metals, nutrients, etc. *Dehalococcoides* was detected at varying POC concentrations. *Dehalococcoides* was detected at very low POC concentrations indicating that *Dehalococcoides* is not dependent on POC as a carbon source for growth and replication. These results are consistent with other studies that have shown that *Dehalococcoides* use only hydrogen as an electron donor and chlorinated compounds as growth-supporting electron acceptors (2, 29, 33).

In every sample where *Dehalococcoides* were detected, hydrogen sulfide was also present (P. Santschi *pers. comm.*). There also seems to be no direct correlation between chlorine, sulfate, iron, or manganese concentrations and the presence of *Dehalococcoides* (P. Santschi *pers. comm.*). However, a trend does appear to exist

between presence of *Dehalococcoides* and age of the sediment. For cores with data available for estimated age of sediments, *Dehalococcoides* is not detectable before 2 years (Table 13). I am still awaiting age data for the rest of the Sea Grant study cores to confirm this trend. If age of the sediments is the overriding factor for establishment of *Dehalococcoides* in the dioxin contaminated sediments, then this means that estimates of natural degradation rates must also take this 'establishment' period into account.

The observation that *Dehalococcoides* was sometimes not found at high dioxin concentrations may be explained by several factors. It is possible that *Dehalococcoides* was present below detection limits. Conventional PCR has a higher detection threshold compared to real-time PCR. *Dehalococcoides* may be detectable in these samples via real-time PCR. Another reason for the absence of *Dehalococcoides* may be patchiness. Perhaps *Dehalococcoides* is not associated with each and every dioxin molecule, and so may not be covering every square centimeter of sediment. Since dioxins are hydrophobic and partition rapidly to organic carbon fractions (i.e. black carbon) (27), they may not be biologically available to bacteria when bound to these fractions. To my knowledge, there are no published studies that investigate whether or not dioxins are biologically available when bound to organic carbon fractions, in particular black carbon. Since I was also unable to find depth profiles of the presence or absence of *Dehalococcoides* in the published literature, the observation that *Dehalococcoides* was sometimes not detected where expected may be due to specific factors that are not yet known.

Dehalococcoides was also detected in both the dredged and undredged samples from the HSC. It occurred in 8 out of 9 samples for the dredged sediment survey and only in 5 out of 9 for the undredged sediment survey. Assuming that there are no false positives these results are surprising since one would expect to find *Dehalococcoides* more in the undisturbed sediment. Overall, the dredged sediment samples had lower dioxin concentrations compared to the undredged samples. This could be due to dredging activity that resuspends dioxins and other chemicals in the water column, thus reducing the dioxin concentrations in the sediment, but increasing them in the water column. Interestingly, *Dehalococcoides* seem to be able to withstand the dredging activity (i.e. mixing and introduction of oxygen) and remain in the sediment. However, since we detected DNA, it is possible that we detected the DNA from dead cells that are not active and growing anymore. Hence we cannot conclude whether or not dredging activity actually kills *Dehalococcoides* or not. It is possible that some bacteria are killed during the dredging activity, but that parts of the microbial communities remain intact. Adrian et al (2000) showed that *Dehalococcoides* strain CBDB1 is extremely oxygen sensitive, this is probably true for all *Dehalococcoides* strains, and if due to dredging activity *Dehalococcoides* were exposed to air, it would most likely die or at the very least become inactive. In conclusion, we detected *Dehalococcoides* in both the dredged and undredged sediment samples from the HSC.

Seven Texas Bay Systems, one additional port, and part of Offatt's Bayou, a tributary to Galveston Bay were also examined for the presence of *Dehalococcoides* (See Ch. I;

Table 16). Sediment grab samples were collected from each system and analyzed for the presence or absence of *Dehalococcoides*. *Dehalococcoides* was only found in two bay systems, Galveston Bay and Sabine Lake. Multiple grab samples were collected from these bay systems and *Dehalococcoides* was not detected in every sample. In Galveston Bay, *Dehalococcoides* was only detected at three stations, Trinity Bay and along the track of the HSC (Ch. I; Fig. 4). It was not detected at the three stations closest to the Gulf of Mexico. This leads to the conclusion that *Dehalococcoides* is migrating out of the HSC into upper Galveston Bay. Either it has not reached the lower bay yet or it is confined to fresher parts of Galveston Bay. Another reason could be that dioxin concentrations are too low in the lower part of Galveston Bay to support *Dehalococcoides* populations. A similar pattern was observed in Sabine Lake, which is also a highly industrialized bay. Again multiple samples were collected from this system and *Dehalococcoides* was only found at the closest to inland stations. *Dehalococcoides* was not found in Port Lavaca or other industrialized bays in Texas, such as Corpus Christi Bay. An explanation could be that grab samples can only collect shallow sediments and we might have missed deeper layers containing *Dehalococcoides* in those systems. Additionally, dioxin concentrations in these bays have not yet been measured and may be too low to support *Dehalococcoides* populations.

These results support the use of *Dehalococcoides* as a biological proxy for dioxin contamination. *Dehalococcoides* was detected at dioxin concentrations ranging from 3 to 239 total TEQ ng/kg dry weight (in HSC sediments), but not at concentrations below 1

total TEQ ng/kg dry weight (wetlands control site). Hence when *Dehalococcoides* was detected in the sediment using molecular methods, the dioxin concentrations were above the background levels of atmospheric deposition. Screening for the presence of *Dehalococcoides* in sediments is a fast and inexpensive way to determine contamination with dioxins. Typical dioxin analysis of sediments in the U.S. costs approximately $1,500, whereas a PCR reaction only costs about $6. Besides being less expensive, screening for *Dehalococcoides* is also faster. Dioxin analysis can take up to several months, whereas PCR analysis can be completed within a few weeks or even days.

V. 1. 2. Bacterial Diversity within HSC Sediments

Sediments in the HSC contained high bacterial diversity as well as a population of *Dehalococcoides*, which dehalogenate various toxic substances, including dioxin. *Dehalococcoides* diversity is centered on *Dehalococcoides ethenogenes* strain 195 and CBDB1 (~17%, combined depths). *Dehalococcoides*-like species comprised between 29% (20cm) and 51% (3cm) of the overall *Dehalococcoides* diversity, indicating that new strains may be evolving. The observation that more *Dehalococcoides*-like species were found at 3cm than at 20cm may indicate that the organisms are deposited from the water column and migrate deeper into the sediments. Since the *Dehalococcoides* clone libraries were constructed from community DNA, some clone sequences may belong to dead organisms, which may very well be the case here, since *Dehalococcoides* are strict anaerobes. Even though *Dehalococcoides* diversity decreased with depth, it did not significantly vary between depths in terms of species present. *Dehalococcoides* sp. strain

CBDB1and *D. ethenogenes* strain 195 have been shown to reductively dechlorinate 1,2,3,4-TCDD (14, 15) and strain CBDB1 is also capable of dechlorinating 1,2,3,7,8-PeCDD (14). Their presence in the HSC sediments points to the dechlorination potential of not only dioxins, but also certain chlorobenzenes (15) and chlorobiphenyls (15, 63).We also found a variety of *Dehalococcoides*-like species which may point to an even greater overall dechlorination potential. It has been speculated that anthropogenic compounds select for microbes that have acquired the ability to use them (53). The diversification of reductive dehalogenase functions in *D. ethenogenes* 195 appears to have been mediated by recent genetic exchange and amplification (53). This leads to the conclusion that the *Dehalococcoides* population is adapting to its surrounding energy sources and that through genetic exchange more species will be able to dechlorinate highly chlorinated compounds, such as dioxins, PCBs, and chlorobenzenes. The HSC sediments may very well be the next "breeding ground" for new *Dehalococcoides* strains.

Overall bacterial diversity is extremely high within HSC sediments. This explosion in diversity may be due to the high level of contamination in the HSC sediments. It seems that bacteria from many different phylogenetic groups, especially *Deltaproteobacteria*, *Dehalococcoides*, and *Firmicutes*, thrive on a wide variety of available substrates. In the HSC sediments *Proteobacteria*, especially *Deltaproteobacteria*, are dominant. They are followed by *Firmicutes* (16.30%). Both *Deltaproteobacteria* and *Firmicutes* are known to have members that are capable of dehalorespiration (53). The *Chloroflexi clade*,

which is closely related to *Dehalococcoides* or is even thought to include *Dehalococcoides,* only represented 5% of the total bacterial diversity. Low coverage of the clone libraries due to poor PCR primer specificity may be the cause, since this clade usually predominates PCB and dioxin contaminated sediments. Difficulties in detecting *Dehalococcoides* in sediments, even laboratory sediment cultures, are not unusual when using bacterial specific primers (63). After confirming the presence of a *Dehalococcoides*-like population in a sediment culture via DGGE (denaturing gradient gel electrophoresis), Yan et al (63) constructed a 16S rRNA clone library which failed to produce sequences that corresponded to the *Dehalococcoides*-like population. After generating a second clone library with Dehalococcoides specific, the correspondence between the *Dehalococcoides*-like DGGE bands and the *Dehalococcoides*-like clones was established based on a perfect and exclusive match.

Total bacterial diversity at the wetlands control site (FW1A) was significantly lower than that observed in the HSC sediments. Almost half of the cloned sequence types detected clustered within the *Proteobacteria* (47%), as compared with 39% in the HSC sediments. The *Betaproteobacteria,* which typically dominate freshwater environments (34), were predominant (29.00%), compared to the *Deltaproteobacteria* (16.3%) in the HSC sediments. The second most abundant group in the FW1A clone library clustered within the phylum *Holophaga/Acidobacteria* (35.50%). There are fewer phylogenetic groups in the wetlands control site, however, diversity based on individual clones was

higher indicating functional specialization within each group. Alternatively, it may be an artifact of a larger sampling size for the wetlands control clone library.

Overall, these results are very promising for (*in situ*) bioremediation in the HSC, since it appears there are a wide variety of bacterial groups present which are able to utilize toxic substances. To my knowledge, this study is the first to examine diversity of dechlorinating bacteria, in particular *Dehalococcoides*, in natural estuarine sediments (not microcosms). In the dioxin contaminated sediments of the HSC *Dehalococcoides* and *Dehalococcoides*-like bacteria were detected; however, none were detected in the wetlands control sediment. This confirms that *Dehalococcoides* require dioxins and other polychlorinated compounds as their terminal electron acceptors, i.e. dehalorespiration. This study also confirms that PCR detection of *Dehalococcoides* using simple 'present/not present' results is a powerful tool to determine contamination of sediments with dioxin or other polychlorinated compounds.

V. 1. 3. Metabolically Active versus Inactive Members of the Microbial Community within the HSC Sediments

Overall bacterial diversity within the sediments of the HSC was extremely high. As shown by the low coverage values of 27 and 21% for the DNA-derived (rDNA) and RNA-derived (rcDNA) clone libraries, respectively, neither clone library sampled bacterial diversity well. Thus we cannot validly conclude that the rcDNA and rDNA clone libraries obtained are truly representative of the active and total bacterial populations within HSC sediments. Poor sampling of species diversity in bacterial

communities using molecular methods is not uncommon. For example, Miskin et al. (36) found 26 and 5% coverage among RNA- and DNA-derived clones, respectively, from anoxic sediments from a productive freshwater lake in England.

Even though only a small fraction of the overall diversity of individual species is represented in these two clone libraries, the major phylogenetic groups (*Proteobacteria* and *Chloroflexi*) are represented well in both. Furthermore, these two major phylogenetic groups are also found in other clone libraries from the HSC sediments (see Ch. III). *Proteobacteria* and *Chloroflexi* make up the majority in both clone libraries, but to varying degrees. In the DNA-derived clone library, *Proteobacteria* account for 50% of the overall diversity, whereas in the RNA-derived clone library, *Proteobacteria* account for 72%. This is a 31% increase and indicates that *Proteobacteria*, especially *Deltaproteobacteria,* are dominant in the metabolically active fraction of the bacterial population in HSC sediments. *Proteobacteria*, in particular *Deltaproteobacteria*, are commonly found among the metabolically active members of microbial populations in a variety of environments, including sediments from an active mud volcano in the Gulf of Mexico (31) and uranium-contaminated subsurface sediments (5). In contrast, *Chloroflexi* account for 11% of the diversity in the DNA-derived clone library and 5% in the RNA-derived one. This is a 55% decrease and indicates that even though *Chloroflexi* are the second most abundant bacterial group in HSC sediments; their metabolically active population is half the size of their total population. Poor biomass yields of *Dehalococcoides* have been reported previously (23). This could be due to several

factors, including localized redox conditions, the biological availability of carbon sources (i.e. dioxins), competition with other dechlorinators (such as *Deltaproteobacteria*), and the presence/ absence of dechlorination stimulators (i.e. other halogenated compounds).

Ahn et al (3), demonstrated the enhancement of reductive dechlorination of 1,2,3,4-TCDD with different coamendments. The study found that halogenated aromatic compounds with structural similarity to 1,2,3,4-TCDD stimulated its dechlorination. Different dechlorination rates were observed with different coamendments, which might have been due to a variety of dehalogenating microorganisms within the different enrichments. This suggests that reductively dehalogenating bacteria may be selected and/or stimulated by specific compounds. Molecular analysis of the bacterial population revealed that distinct microbial populations, namely *Chloroflexi*-like microorganisms related to *Dehalococcoides*, were enriched with halogenated coamendments (4). Yan et al (63) found that the addition of different electron donors (Fe (0) and a mixture of fatty acids) to sediment cultures from Baltimore Harbor resulted in differences in the lag period prior to dechlorination and in the extent of dechlorination. Dechlorination of 2,3,4,5-tetrachlorobiphenyl (CB) to 2,3,5-CB occurred in both cultures, however, further dechlorination to 2,5-CB only occurred in the fatty acid amended culture.

The remaining phylogenetic groups such as *Holophaga/Acidobacteria*, *Spirochetes*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, and various Candidate Divisions follow a

pattern similar to that of *Chloroflexi*. These groups combined make up roughly 40% of the overall diversity in the DNA-derived clone library, but only 23% in the RNA-derived one. This is a 42% decrease and indicates that the metabolically active populations of these groups are significantly smaller than their total populations. Overall, this shows how the metabolically active fraction in HSC sediments is dominated by the *Deltaproteobacteria* (41%). Just like *Chloroflexi*, members of the *Deltaproteobacteria* are also capable of dechlorination (for example, *Desulfomonile* and *Desulfuromonas*) as well as sulfate reduction (22, 33). Again, it is noteworthy that almost all the phylotypes from both clone libraries are most closely related to other uncultured phylotypes from (contaminated) sediments worldwide. This phenomenon is often reported for 16S rRNA gene sequences recovered from environmental samples (31, 36).

The observation that some rcDNA (RNA) phylotypes were not detected in the rDNA (DNA) clone library might be explained by either small DNA fragments (a result of the extraction method) and/or the low coverage of the rDNA clone library. Since the rDNA clone library represents the total bacterial population and the rcDNA clone library represents the metabolically active bacterial population, one would expect every phylotype in the rcDNA clone library to be present in the rDNA clone library as well. Since the extraction method included a bead beating step it is possible that some genomic DNA was fragmented during the extraction process. The recovery of smaller nucleic acid fragments is not uncommon (36). Coverage for the rDNA and rcDNA clone libraries was 27 and 21%, respectively. These coverages are rather low and indicate poor

sampling of species diversity. Larger clone libraries are needed to sample bacterial diversity well and validly conclude that the rcDNA and rDNA clone libraries obtained are truly representative of the active and total bacterial populations within HSC sediments.

This is the first study to report the composition of the metabolically active members of the bacterial community in HSC sediments. Even though phylogenetic surveys derived from DNA are informative, they cannot determine the ecological significance of the organisms from which the gene sequences were recovered, since DNA is known to persist in dead cells and extracellularly (36). RNA, on the other hand, is highly labile and rRNA levels have been correlated with cellular activity (35). Sequences recovered from an RNA template using RT-PCR imply that the source organisms were active *in situ* at the time of sampling or close to it (31, 36, 41). This is in contrast to DNA templates where such a distinction cannot be made (35). Using rRNA as a means to study the metabolically active members of the bacterial community has been proposed for several years (36). Unfortunately, RNA is more difficult to isolate than DNA. This is due mainly to the rapid degradation of RNA by the enzyme RNase, which is both stable and ubiquitous (36). To date, most methods developed for RNA extraction from sediments have been rather lengthy and some also require expensive equipment (e.g. an ultracentrifuge). Miskin et al (36) developed a rapid method for the extraction of RNA from sediments; however, the developed method, which used sterile glass beads (0.17-0.18 mm diameter), only yielded reproducible rRNA fragments of about 530 base pairs.

Such short fragments make phylogenetic analysis difficult. The RNA extraction method used in this study yielded rRNA fragments of about 1,100 base pairs, making phylogenetic analysis easier and more reliable. In the end, every RNA extraction method has its own biases and trade-offs and choosing the best method is always a difficult decision.

Although the bacterial composition determined by this RT-PCR approach is not quantitative, it does suggest that the organisms represented by these sequences play an important metabolic role in the sediments (41). Hence, these results are promising with respect to *in situ* bioremediation in HSC sediments. This study confirms that members of the group *Chloroflexi*, which includes *Dehalococcoides*, are indeed part of the metabolically active fraction of the bacterial community in HSC sediments. Since these organisms are already present and active, it would make *in situ* bioremediation more feasible and successful. It would be interesting to construct and analyze a RNA-derived clone library targeting the bacterial group *Dehalococcoides*. Such a clone library would shed more light on the active members of the bacterial community who are able to dechlorinate halogenated compounds, such as dioxin.

Another possibility would be to specifically target reductive dehalogenase (RD) genes. These genes have been shown to be responsible for the dechlorination of halogenated aromatic compounds (23, 53). A variety of different RD genes have been identified (23, 53). 17 putative RD genes were identified in the genome of *Dehalococcoides*

ethenogenes strain 195 (53). Thirty novel RD genes have been amplified from *Dehalococcoides* sp. strain CBDB1 and *Dehalococcoides* sp. strain FL2, and they all differed from the 17 RD genes identified in the genome of *D. ethenogenes* strain 195 (23). However, the RD gene responsible for the dechlorination of dioxins has yet to be identified. The presence of multiple nonidentical RD genes in *Dehalococcoides* strains is consistent with the observation that the different strains use different chlorinated compounds as their electron acceptors (2, 14, 29). It has been suggested that the presence of multiple RD genes is most likely due to older evolutionary events and not due to rapid adaptation to the presence of anthropogenic halogenated compounds (23). However, lateral gene transfer is highly likely and may facilitate the adaptation to new environments. Hence, rather than evolving new genes, *Dehalococcoides* strains may simply look to their neighbor for new dechlorination capabilities. Knowing, in more detail, which *Dehalococcoides* phylotypes and RD genes in particular are the most active within the HSC sediments will be helpful in determining which strains to focus on for *in situ* bioremediation.

V. 1. 4. Overall Conclusions

PCDDs and PCDFs are widespread persistent organic pollutants. Due to their hydrophobicity they tend to partition on to the organic fractions (i.e. black carbon) in soils and sediments (8, 27). Dioxins can cause adverse health effects in fish, wildlife, and humans (7, 58). Studies have shown that hydrophobic, fat-soluble substances with high octanol-water partition coefficients (K_{OW}) ($\geq$100,000) such as PCDDs and PCDFs

biomagnify not only in aquatic food webs but also terrestrial ones (21, 26) Therefore remediation of contaminated sediments is very important. One very promising *in situ* process is microbial reductive dechlorination, as it has the potential of decreasing the toxicity of PCDDs and PCDFs (3, 14, 15).

The Port of Houston is the sixth largest seaport in the world and handles more foreign water-borne tonnage than any other U.S. port. Each year more than 6,300 vessels pass through the HSC, making it a highly industrialized area. Both the HSC and upper Galveston Bay (GB) are highly polluted with dioxins, dioxin-like compounds, and many other contaminants, such as hydrocarbons, from industrial and municipal effluents and runoff, as well as from atmospheric wet and dry deposition (57). Because the HSC is so highly polluted, it is a prime site for *in situ* bioremediation.

The main objective of this study was to determine the potential for *in situ* bioremediation in the HSC sediments. Our study focused on the bacterial group *Dehalococcoides*, since it is the only known group to reductively dechlorinate dioxins (14, 15). *Dehalococcoides* was detected within HSC sediments and its distribution seems to be rather extensive. It appears that *Dehalococcoides* needs four things to occur in the HSC sediment:

1.) greater than 3 TEQ ng/kg dry weight dioxin concentrations.

2.) presence of detectable hydrogen sulfide.

3.) sediment which has accumulated for 2 years or more

4.) presence of POC, at greater or equal to 0.4%.

(this actually indicates that *Dehalococcoides* is independent of POC concentration)

The presence of *Dehalococcoides* in the HSC, the upper part of GB, and Sabine Lake is very promising in terms of bioremediation. Incubation studies with environmental samples have shown that *Dehalococcoides* can dechlorinate dioxins and other chemicals, such as PCBs (1, 2, 9, 14, 15, 22). Incubation experiments with dioxin contaminated HSC sediments are currently underway at TAMUG using various carbon sources to accelerate dechlorination rates and should provide insights for accelerating *in situ* bioremediation throughout the HSC.

Sediments in the HSC contained high bacterial diversity as well as a population of *Dehalococcoides* and *Dehalococcoides*-like organisms. *Dehalococcoides* diversity was centered on *Dehalococcoides ethenogenes* strain 195 and *Dehalococcoides* sp. strain CBDB1. *Dehalococcoides* diversity decreased with depth, but did not significantly vary with depth in terms of species present. Overall bacterial diversity was extremely high in HSC sediments. This explosion in diversity may be due to the high level of contamination in the HSC sediments. It seems that bacteria thrive on all sorts of substrates there. In the HSC sediments *Proteobacteria*, especially *Deltaproteobacteria*, and *Chloroflexi*-like organisms, including *Dehalococcoides,* were the most dominant groups. On the other hand, *Betaproteobacteria* and *Acidobacteria* were dominant in the wetlands control sediment. There were fewer phylogenetic groups in the wetlands control site, but diversity based on individual clones was higher. Almost all of the cloned

sequence types were most closely related to other uncultured organism found in a wide variety of environments. A closer look at the metabolically active members of the microbial community in the HSC sediments revealed that most phylogenetic groups detected via clone libraries originating from DNA samples are active members of the community. In particular, *Deltaproteobacteria* and *Chloroflexi* are the most active groups in HSC sediments. Overall, these results are very promising for *in situ* bioremediation in the HSC, since it appears there are already bacterial groups present that are able to utilize toxic substances. To my knowledge, this study is the first to examine diversity of microbial communities in natural estuarine sediments (not microcosms) contaminated with dioxins. *Dehalococcoides* and *Dehalococcoides*-like bacteria were detected in the dioxin contaminated sediments of the HSC; however, none were detected in the wetlands control sediments. These findings confirm that *Dehalococcoides* require dioxins and other polychlorinated compounds as their terminal electron acceptors, i.e. dehalorespiration. This study also confirms that PCR detection of *Dehalococcoides* using simple 'present/not present' results is a powerful tool to determine contamination of sediments with dioxin and/or other polychlorinated compounds. Since it appears that *Dehalococcoides* need a minimum of 3 TEQ ng/kg dry weight, this tool will also be helpful in determining where more extensive analyses are needed in terms of dioxin contamination. There is no question that the HSC is in dire need of remediation of its contaminated sediments and *in situ* bioremediation, which utilizes the reductive dechlorination capabilities of *Dehalococcoides*, is very likely the best way to accomplish this task.

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VITA

Name:	Anne-Sophie Charlotte Hieke
Address:	1505 Ingram Terrace, Silver Spring, MD 20906
Email Address:	charlotte_hieke@neo.tamu.edu
Education:	B.S., Marine Biology, Texas A&M University, 2005